FY2002 FINAL REPORT

Ecological Risk Assessment of Explosive Residues in Rodents, Reptiles, Amphibians, and Fish

SERDP Project ER-1235

March 2004

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14. ABSTRACT

Little information is available regarding the uptake kinetics of RDX or HMX by typical wetland (capable of root penetration into anaerobic zones) plants in constructed or real wetland systems. RDX uptake in aquatic and wetland plants has been studied previously (e.g. Best et al., 1997) but has not been rigorously explored. Wetlands are a key interface between non-point source runoff (e.g. firing ranges) and surface water or groundwater. Uptake kinetics are critical for an overall understanding of exposure as real systems are typically transiently loaded and concentration profiles are variable with depth due to microbial degradation. Leaching of RDX from simulated rain events and from simulated seasonal or event flooding will also play an important role in overall fate and exposure.

15. SUBJECT TERMS

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FINAL REPORT FY2002
SERDP Project: ER-1235

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29 MAR 2004

IDENTIFICATION OF PERCHLORATE-CONTAMINATED AND REFERENCE SITES IN AND AROUND THE NAVAL SURFACE WARFARE CENTER IN INDIAN HEAD, MARYLAND

STUDY NUMBER:	IHM-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	October 3, 2003
RESEARCH COMPLETION:	December 31, 2003

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

James A. Carr

3/26/04
Date

1.0 DESCRIPTIVE STUDY TITLE: Identification of perchlorate-contaminated and reference sites in and around the Naval Surface Warfare Center in Indian Head, Maryland.

2.0 STUDY NUMBER: IHM-03-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
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Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: 10/3/03
Termination Date: 12/31/03

6.0 KEY PERSONNEL:
James A. Carr, Co-Principal Investigator/ DBS Testing Facility Management/Study Director
Mike Wages, Research Associate
Todd Anderson, Analytical Chemist/ Asst. Director for Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator/ TIEHH Testing Facility Management

7.0 STUDY SUMMARY:
We collected water samples from eleven surface water sites in and around the Indian Head NSWC during two trips in October 2003. Water quality parameters of the surface waters were determined and samples from all sites were analyzed for perchlorate content to determine potential reference and contaminated sites for future study. Of the eleven sites examined, five contained measurable perchlorate concentrations ranging from 45 to greater than 550 µg/L perchlorate. All of the perchlorate contaminated sites were found on the military base; no perchlorate was detected offsite. Most of the perchlorate-contmainated sites contained tadpoles. Our data suggest that that both reference and perchlorate-contaminated sites exist in the vicinity of the Indian Head NSWC, and provide a basis for future basis for future investigations on natural amphibian populations inhabiting perchlorate-contaminated surface waters.

8.0 METHODS:

Sites were selected based on their proximity to perchlorate manufacturing sites within the NSWC. At each visit to the field site, we measured various compositional and physical parameters, including:

1. dissolved oxygen
2. water temperature
3. conductivity
4. total dissolved solids
5. pH

During each visit, a water sample was collected from the water body. Waters were collected by hand in clean glass 20 mL scintillation vials (AQ-3-03) and kept cool (i.e. in boxes and/or in shade) during transport to our laboratory where they were stored at approximately 4 °C until analyzed for perchlorate (AC-2-11). We attempted to visit each site twice during the fall of 2003.

9.0 RESULTS:

The Indian Head NSWC is an operational weapons testing and development facility that is located approximately 25 miles south of Washington D.C. (Figure 1). The Indian Head peninsula is bordered on the west by the Potomac River and on the east by Mattawoman Creek. The NSWC continues to mill and burn perchlorate, and was the subject of an in-depth study on perchlorate biotransport in 2001(Parsons Engineering Science, Inc., 2001). Our goal was not to systematically sample every surface water body at the NSWC, but to focus on those sites with a high probability of serving as a habitat for local anurans. Others (Parsons Engineering Science, Inc., 2001) have compiled a more thorough sampling of flora and fauna associated with perchlorate contaminated water bodies at NSWC. Our collections focused around a perchlorate milling site in the center of the facility (Low Vulnerability Ordnance Ammunition, LVOA, Area) that drains into a series of marshes and beaver ponds collectively called the Town Gut Marsh ponds. The Town Gut Marsh ponds drain in turn into Mattawoman creek (Figure 2).

Site IDs and locations are presented in Table 1. The physical characteristics of the collection sites are presented in Tables 2-12. Our initial effort focused on the LVOA area (IW85), which contained a small pond inhabited by tadpoles. There is a perchlorate grinding facility in close proximity to this pond. As shown in Tables 2-12, perchlorate was detected in all of these sites, from IW85 south to MCRN, which lies at the intersection of Noble road and Mattawoman creek. Moreover, perchlorate concentrations were consistently lower at the sites south of IW85, a finding that is consistent with the notion that perchlorate moves with surface water drainage from the LVOA south to the Town Gut Marsh system and into Mattawoman creek.

We focused on sites off of the naval base as potential reference sites. These included a site at Mattawoman creek off of highway 225 and a boat marina in Smallwood State Park. Neither site contained measurable perchlorate. Additionally, three sites were identified on base at the NSWC with no measurable perchlorate. These were a golf course pond off of Strauss Avenue (SA), the Potomac River at the NSWC marina (SPD), and a small holding pond at the marina (MSP).

Table 1. Site information for collection of water samples in and around the Naval Surface Warfare Center in Indian Head, Maryland.

Site ID	Location	GPS (UTM)	Water Samples A & B	Water Samples C & D
AR	Atkins Road	18S 0309714 4272656	10/3/03	10/16/03
ARSN	Atkins Road Spur - North	18S 0309811 4272828	10/3/03	10/16/03
ARSS	Atkins Road Spur - South	18S 0307910 4272985	10/3/03	10/16/03
IW85	Adjacent to building 1185	18S 0311225 4275280	10/3/03	10/16/03
MARINA	MARINA**	18S 0309898 4274681	10/3/03	NC
MCNR	Noble Rd & Mattawoman Creek	18S 0309722 4272352	10/3/03	10/16/03
MSP	Marina Settling Pond (IW48)	18S 0309970 4274697	10/4/03	10/16/03
MWC	Mattawoman Creek & Hwy 225*	18S 0315689 4273493	10/4/03	10/16/03
SA	Strauss Ave Golf Pond	18S 0309875 4273956	NC	10/16/03
SPD	Potomac River Boat Dock**	18S 0309269 4270028	10/4/03	10/16/03
SPM	Sweden Point Marina **	18S 0309604 4269939	10/4/03	10/16/03

* Northeast of intersection of Hwy 225 & Hwy 224
** In Smallwood State Park
*** In IHDNSWC

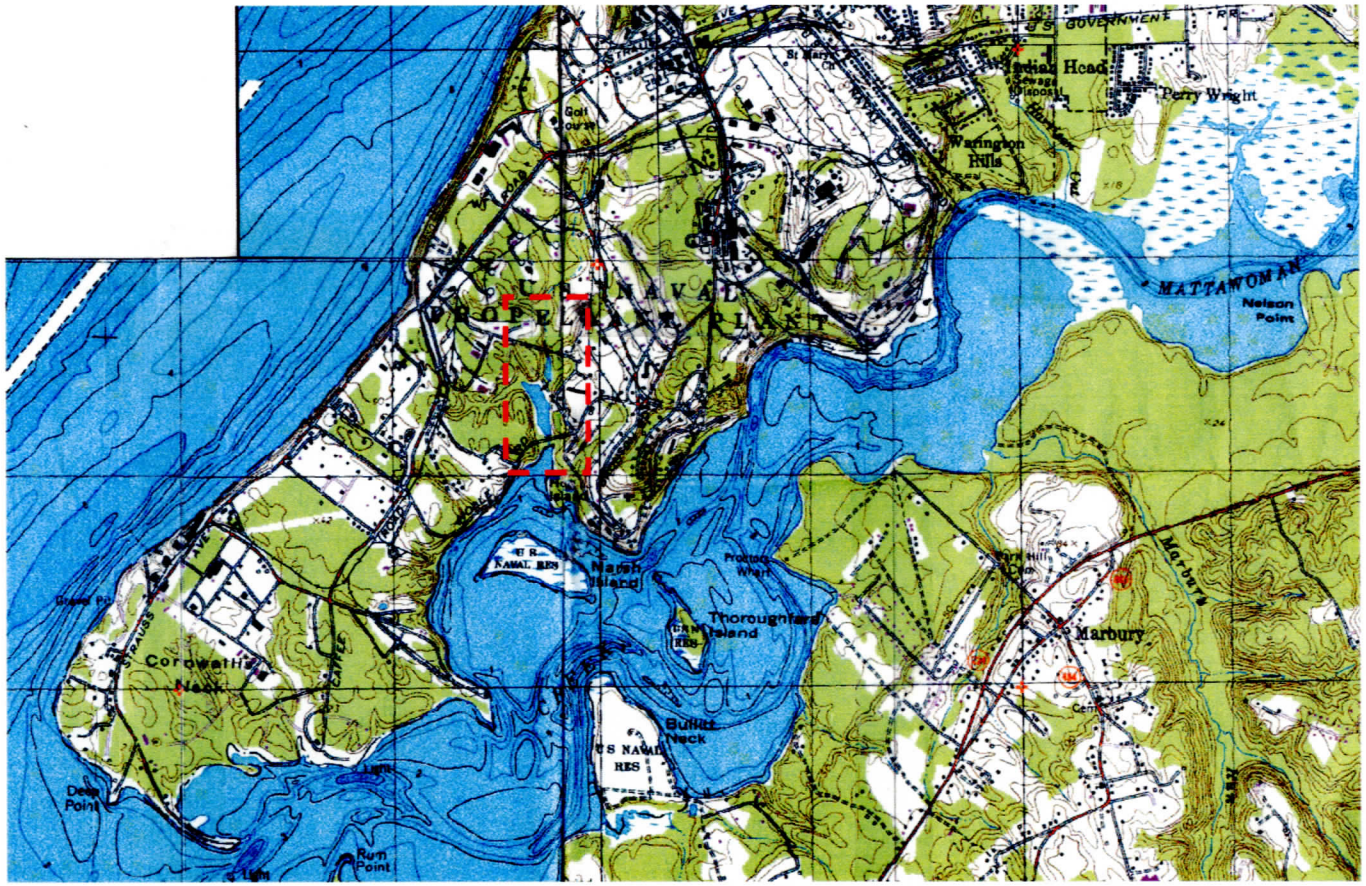

Figure 1. Map of the Naval Surface Warfare Center in Indian Head, Maryland. The sites IW85, ARSN, ARSS, AR, and MCNR are located in the red box and are enlarged in Figure 2.

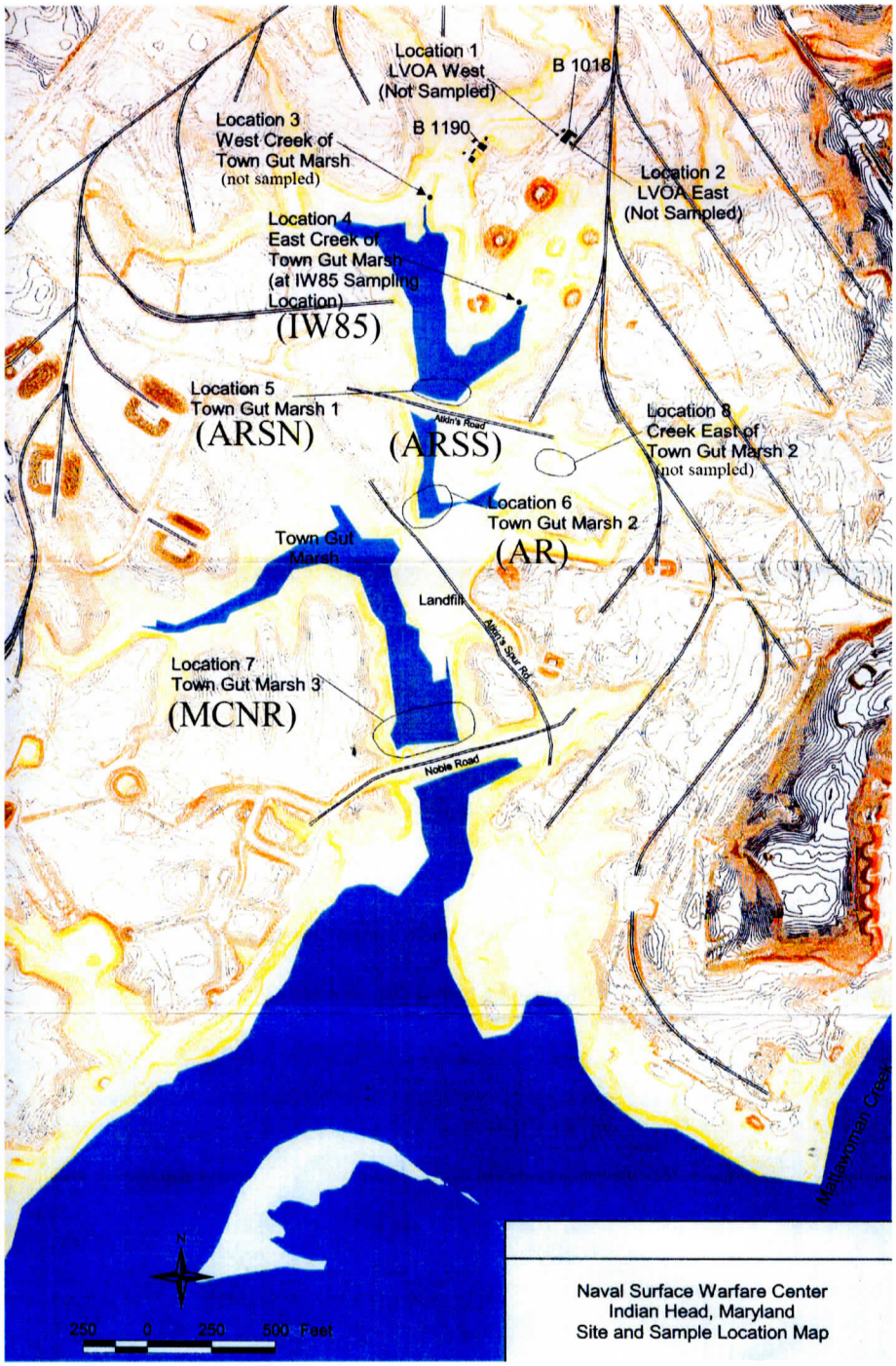

Figure 2. Enlarged view of the sites (ARSN, ARSS, AR, and MCNR) located just south of the collection site (IW85) in closest proximity to the low vulnerability ordnance ammunition area (LVOA). The buildings in the LVOA area are used for milling perchlorate. Sites IW85, AR, ARSS, ARSN, and MCNR are all part of the Town Gut Marsh pond system. Surface water flows south from the LVOA through the Town Gut Marsh system into Mattawoman creek. The Potomac River lies just west of the facility and is not shown in this enlarged site map. Modified from an original map by Parsons Engineering Science, Inc. (2001).

Table 2. Physical properties of site IW85

Grid reference	18S 0311225 4275280
Water temperature (°C)	13.6
pH	5.85
Dissolved oxygen (mg/L)	4.98
Conductivity (µS/cm)	329
Total dissolved solids (g/L)	0.21
Perchlorate (µg/L)	463-555*

*range of values based on two sampling dates.

Table 3. Physical properties of site ARSN

Grid reference	18S 0309811 4272828
Water temperature (°C)	14.3
pH	6.55
Dissolved oxygen (mg/L)	4.98
Conductivity (µS/cm)	277
Total dissolved solids (g/L)	0.18
Perchlorate (µg/L)	99-131*

*range of values based on two sampling dates.

Table 4. Physical properties of site ARSS

Grid reference	18S 0307910 4272985
Water temperature (°C)	15.9
pH	6.56
Dissolved oxygen (mg/L)	3.44
Conductivity (µS/cm)	223
Total dissolved solids (g/L)	0.15
Perchlorate (µg/L)	55-64*

*range of values based on two sampling dates.

Table 5. Physical properties of site MCNR

Grid reference	18S 0309722 4272352
Water temperature (°C)	18.1
pH	6.91
Dissolved oxygen (mg/L)	5.56
Conductivity (µS/cm)	129
Total dissolved solids (g/L)	0.08
Perchlorate (µg/L)	45-121*

*range of values based on two sampling dates.

Table 6. Physical properties of site AR.

Grid reference	18S 0309714 4272656
Water temperature (°C)	16.8
pH	7.05
Dissolved oxygen (mg/L)	5.43
Conductivity (µS/cm)	208
Total dissolved solids (g/L)	0.14
Perchlorate (µg/L)	57-95*

*range of values based on two sampling dates.

Table 7. Physical properties of site MWC.

Grid reference	18S 0315689 4273493
Water temperature (°C)	16.1
pH	6.23
Dissolved oxygen (mg/L)	3.74
Conductivity (µS/cm)	54
Total dissolved solids (g/L)	0.04
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

Table 8. Physical properties of site SPM.

Grid reference	18S 0309604 4269939
Water temperature (°C)	15.9
pH	6.85
Dissolved oxygen (mg/L)	2.92
Conductivity (µS/cm)	81
Total dissolved solids (g/L)	0.05
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

Table 9. Physical properties of site SA.

Grid reference	18S 0309875 4273956
Water temperature (°C)	15.4
pH	6.84
Dissolved oxygen (mg/L)	5.09
Conductivity (µS/cm)	221
Total dissolved solids (g/L)	0.14
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

Table 10. Physical properties of site MSP.

Grid reference	18S 0309970 4274697
Water temperature (°C)	16.4
pH	8.16
Dissolved oxygen (mg/L)	6.33
Conductivity (µS/cm)	193
Total dissolved solids (g/L)	0.13
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

Table 11. Physical properties of site SPD.

Grid reference	18S 0309269 4270028
Water temperature (°C)	21.5
pH	7.16
Dissolved oxygen (mg/L)	5.8
Conductivity (µS/cm)	119
Total dissolved solids (g/L)	0.08
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

Table 12. Physical properties of site Marina.

Grid reference	18S 0309898 4274681
Water temperature (°C)	Not sampled
pH	Not sampled
Dissolved oxygen (mg/L)	Not sampled
Conductivity (µS/cm)	Not sampled
Total dissolved solids (g/L)	Not sampled
Perchlorate (µg/L)	ND*

* Non-detectable based on two sampling dates.

10.0 DISCUSSION:

Our data indicate that surface waters in the vicinity of the LVOA contain measurable perchlorate concentrations in the range of 45 μg perchlorate/L to more than 550 μg perchlorate/L. No perchlorate was detected west of the LVOA in the Potomac River, nor was perchlorate detected at any of the surface water sites located in Mattawoman creek off of the NSWC. The results for perchlorate levels at site IW85 agree with a previous report to the degree that in the previous report perchlorate was detected in 6 of 6 samples from this site. In the previous study surface water levels of perchlorate at this site ranged from 22-24 μg perchlorate/L (Parsons Engineering Science, Inc., 2001), whereas in the present study perchlorate levels in surface water at this site ranged from 463-555 μg perchlorate/L. Likewise, we detected perchlorate at sites AR, ARSN, ARSS, and MCNR, all of which are part of the Town Gut Marsh system. Sites ARSN, AR, and MCNR correspond to sampling sites 5, 6 and 7 as reported by Parsons Engineering Science (2001), all of which were reported to contain measurable perchlorate in surface waters in 1005 of the samples analyzed. In the previous study perchlorate concentrations at sites 5,6, and 7 range from 12 μg perchlorate/L at site 7 (the farthest away from the LVOA) to 25 μg perchlorate/L at site 6 (Parsons Engineering Science, Inc., 2001). Thus, several sites with likely anuran populations at the Indian Head NSWC contain measurable perchlorate as determined by two separate analyses in 2001 and 2003. We were unable to detect perchlorate in any of the sites located off base, suggesting that any of these sites with viable anuran populations may serve as reference sites.

11.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

12.0 REFERENCES:

Parsons Engineering Science, Inc. (2001). Interim final scientific and technical report for perchlorate biotransport investigation. A study of perchlorate occurrence in selected ecosystems. Report contract #F41624-95-D-9018.

29 MAR 2004

TITLE: Effect of ammonium perchlorate on thyroid function of zebrafish

STUDY NUMBER:	ZEB-02-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
ANIMAL TEST SITE:	Texas Cooperative Fish and Wildlife Research Unit Texas Tech University Box 2120 Lubbock, TX 79409-2120
RESEARCH INITIATION:	06-15-02
RESEARCH COMPLETION:	12-31-2003

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Figure 3. Changes in hypertrophy and angiogenesis of zebrafish thyroid follicles during recovery from exposure to AP-derived perchlorate at measured concentrations 0 to 11480 ppb for 12 weeks. **Page 23**

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Reynaldo Patiño

Reynaldo Patiño

3-29-04

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 25, 2004
Date

1. Descriptive Study Title:
Effect of ammonium perchlorate on thyroid function of zebrafish

2. Study Number:
ZEB-02-01

3. Sponsor:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. Testing Facility Name and Address:
Texas Cooperative Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. Proposed Experiment Start and Termination Dates:

Start date:	06-15-2002
Termination Dates:	12-31-2003

6. Key Personnel:
Reynaldo Patiño, Testing Facility Management
Sandeep Mukhi, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. Study Objectives/Purpose:

1. To determine the concentration and time-dependent effects of ammonium perchlorate on thyroid follicle histopathology.
2. To determine the time-course of thyroid histopathological recovery following the termination of ammonium perchlorate exposure.
3. To determine the concentration and time-dependent effects of ammonium perchlorate on body growth (weight and fork length) and condition factor.
4. To determine the effect of perchlorate on whole-body thyroxin content.
5. To determine the relative usefulness of the measured traits as biomarkers of ammonium perchlorate exposure.

8. Study Summary

Perchlorate is an environmental contaminant of increasing concern. Its concentration in contaminated ground and surface waters is commonly reported to be ≤ 100 ppb. A major source

of environmental perchlorate is ammonium perchlorate (AP). Perchlorate inhibits iodide uptake by thyroid follicles and lowers their production of thyroid hormones (e.g., thyroxin). This leads to increased secretion of thyroid-stimulating hormone by the pituitary gland, which consequently causes hyper-stimulation or hypertrophy of thyroid follicles. Hypertrophy is widely used marker of thyroid activity. However, its usefulness as maker of exposure to environmentally relevant concentrations of perchlorate for field surveys or laboratory tests has not been fully validated. Therefore, the objective of this study is to evaluate and compare several biomarkers of perchlorate exposure using zebrafish as animal model. We focused on six different biological endpoints as possible markers of perchlorate exposure: thyroid follicle angiogenesis, thyroid follicle hypertrophy, thyroid follicle colloid depletion, whole-body thyroxin concentration, body growth (weight and fork length), and condition factor. Immature (3-month-old) zebrafish were exposed to AP at measured perchlorate concentrations of 0, 10, 90, 1131 and 11480 ppb for 12 weeks, and then allowed to recover in clean water for an additional 12 weeks. Sampling was conducted at 2, 4, 8 and 12 weeks of exposure and at 4 and 12 weeks after exposure. At 2 weeks of exposure, thyroid follicle angiogenesis was the only marker for which change was recorded with a lowest observed effective level (LOEL) of perchlorate of 90 ppb. Hypertrophy was first noted at 4 weeks of exposure with a LOEL of 11480 ppb, and colloid depletion at 8 weeks with a LOEL of 11480 ppb. By 12 weeks of exposure, the LOEL for angiogenesis remained at 90 ppb and for hypertrophy and colloid depletion had simultaneously decreased to 1131 ppb. Thus, a time-dependent decline in LOEL was observed for hypertrophy and colloid depletion but not for angiogenesis. At the completion of the 12-week recovery period, residual effects on angiogenesis were still present (LOEL, 11480 ppb), whereas hypertrophy and colloid depletion were no longer evident. Whole-body thyroxin concentration, body growth and condition factor were not affected by AP exposure at any time or at any of the concentrations tested. Also, the experimental fish became sexually mature by the end of the experimental period regardless of treatment. In conclusion, this study showed that angiogenesis is a much more sensitive marker of perchlorate exposure than the other parameters examined. Namely, angiogenesis not only responded faster and at lower concentrations of perchlorate, but also persisted for longer periods of time after removal of perchlorate from the water. Further, the LOEL for angiogenesis is within the environmentally relevant range of perchlorate concentrations.

9. Test Materials:

Test Chemical name: Ammonium perchlorate
CAS number: 7790-98-9
Characterization: white powder: specific gravity of 1.950
Purity: Certificate of analysis will be obtained from the company. This chemical will be 99.999% pure as indicated by supplier.
Stability: incompatible with strong reducing agents, strong acids, heat-sensitive. The ammonia is a hazardous combustion or decomposition product.
Source: Aldrich Chemical Co., Inc.

Reference Chemical name: Calcium Chloride
CAS number 10035-04-8
Characterization: coarse white powder or mixture with medium size granules.
Purity: certificate of analysis or analytical testing will indicate purity.

Stability: stable
Source: Sigma

Reference Chemical name: Magnesium Sulfate
CAS number: 100-34-99-8
Characterization: colorless crystals
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Potassium Chloride
CAS number: 7447-40-7
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sea Salts
CAS number: Not applicable
Characterization: an artificial salt mixture closely resembling the composition of the dissolved salts of ocean water.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Aquarium Systems, Inc.

Reference Chemical name: Sodium Bicarbonate
CAS number: 144-55-8
Characterization: white crystalline powder
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sodium Chloride
CAS number: 7647-14-5
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Ultrapure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.
CAS Number: Not applicable
Characterization: water quality will be tested by chemical analysis and pH will be monitored regularly.

Purity: ultrapure
Stability: stable
Source: City tap water or steam plant deionized water that has been run through a carbon filter and a de-ionizer to convert it to ultrapure water. Sea salts (60-240 mg/L) will be added to this water, or a modified FETAX will be made.

Reference Chemical name: L- [^{125}I]-thyroxin
CAS number: not applicable
Characterization: In presence of excess antibody total binding of tracer should be 70-100%. See SOP IN-2-04-01 for detail.
Purity: Initially greater than 95% radiochemically pure as determined by HPLC (Technical data sheet from manufacturer).
Stability: half-life-60days
Source: Perkin-Elmer Life Sciences, Inc. Boston, MA, USA).

Reference Chemical name: Thyroxin antiserum
CAS number: not applicable.
Characterization: 100% reactivity towards L- and D-thyroxin, 1.2% cross reactivity with L-triiodothyronine (T_3), very low cross reactivity with mono or di-iodo-L-tyrosine (<0.001%) at 50% displacement.
Purity: crude antiserum preparation (rabbit anti-thyroxin BSA serum).
Source: ICN Biomedicals, Inc. (Costa Mesa, CA, USA).

10. Justification of Test System:

Perchlorate alters thyroid gland function by inhibiting sodium-iodide symporters that regulate the amount of iodide taken into thyroid follicles (Wolff, 1998). Reduced uptake of iodide suppresses the production and consequently the release of thyroid hormones thereby reducing their levels in blood. This condition causes the pituitary gland to increase its production of thyroid-stimulating hormone (TSH) in an attempt to restore thyroid hormone production and levels. Prolonged exposures to perchlorate causes hyper-stimulation of the thyroid gland (Wolff, 1998; Soldin et al., 2001). Histopathological studies have revealed that exposure to AP-derived perchlorate leads to thyroid follicle hypertrophy, hyperplasia, and colloid depletion in a number of vertebrates (Fernandez Rodriguez et al., 1991; Siglin et al., 2000; York et al., 2001b; Goleman et al., 2002a), including zebrafish (Patiño et al., 2003a). Thyroid follicle angiogenesis has also been observed in AP-treated rats (Fernandez Rodriguez et al., 1991) and zebrafish (Patiño et al., 2003a).

Thyroid follicle hypertrophy is among the most widely used histological markers of thyroid activity, but its usefulness as maker of exposure to environmentally relevant concentrations of perchlorate for field surveys or laboratory tests has not been fully validated. Similarly, although thyroid follicle angiogenesis is a remarkable histological event in adult zebrafish exposed to AP (Patiño et al., 2003), its behavior as maker of perchlorate exposure has not been explored. The objective of this study is to evaluate and compare several markers of perchlorate exposure using zebrafish as animal model. We focused on six different biological endpoints as possible markers

of perchlorate exposure: thyroid follicle angiogenesis, thyroid follicle hypertrophy, thyroid follicle colloid depletion, whole-body thyroxin concentration, body growth (weight and fork length), and condition factor.

Zebrafish was used as experimental animal because it is a widely used animal model in basic toxicological, developmental and reproductive research. This species is also easily and economically maintained in the laboratory. Further, our earlier experiments that generated the questions being addressed by the present study were conducted with zebrafish.

11. Test Animals:

Species:	*Brachydanio rerio*, Zebrafish
Strain:	Wildtype
Age:	Sub-adults (2 months of age at the time of purchase).
Number:	675, mixed sex population.
Source:	Fish were purchased from Aquatic Research Organisms (Hampton, NH, USA)

12. Procedure for Identifying the Test System:

Upon arrival from the vendor, 2-month-old zebrafish of unknown sex were randomly assigned to pre-cleaned (SOP AF-1-01) 10-gallon glass aquaria filled with 30 liters of zebrafish water at approximately 50 fish per tank. They were allowed to acclimatize to the laboratory for 4 weeks before the exposures were started, and were therefore approximately 3-month-old at the onset of exposures. Tanks were appropriately labeled with a minimum of the following: project number, initials, concentration, chemical, replicate number, and date initiated. Treatment solutions were added to the appropriate tanks according to SOP AF-1-01.

The following tasks were conducted:

Task 1 (exposure and recovery): Fish were exposed to nominal concentrations of 0, 10, 100, 1000 and 10000 ppb of AP according to SOP-AF-1-01.

Task 2 (histological sample collection): Samples for histological analysis were taken at 2, 4, 8 and 12 weeks of exposure and at 4 and 12 weeks after removal of AP.

Task 3 (whole-body thyroxin content): Samples for whole-body thyroxin content analysis were taken at 6 and 12 weeks of exposure and at 12 weeks after removal of AP.

Fork lengths and weights were recorded on all samples taken. Fish were fed with commercial diet twice daily. Feeding behavior and general health were monitored by daily observations. Water temperature, pH, salinity, and dissolved oxygen were recorded daily. One half of the water volume in the aquaria (15L) was replaced once weekly and ammonia level was also monitored once weekly. At each sampling time, fish were euthanized in a lethal concentration of anesthetic (1g/L MS-222). Following an abdominal incision, fish collected for histological analysis were placed in Bouin's solution, and further processing was followed according to SOP

AQ-2-10. Fish taken for thyroxin content analysis were flash-frozen in liquid nitrogen and stored at - 80 °C.

13. Experimental Design Including Bias Control:

After the acclimation period, ammonium perchlorate was added to the tanks at final nominal concentrations of 0, 10, 100, 1000, and 10000 ppb (five treatments). Three replicate aquaria per AP concentration were prepared housing a total of 750 fish. At each sampling time, 5 fish were sampled from each replicate. There were a total of 6 sampling times during the exposure and recovery periods for histological analysis, and 3 sampling times for whole-body thyroid hormone content.

The total fish required for histology is calculated as: 5 treatments X 3 tank replicates per treatment X 6 sampling times X 5 fish per sampling time = 450 fish. The total fish required for thyroxin analysis is: 5 treatments X 3 tank replicates per treatment X 3 sampling times X 5 fish per sampling time = 225. Therefore, the total number of fish sampled for analysis was 675 (50 in each of 15 aquaria).

14. Methods

14.1 Animal Husbandry

The use of animals in this study was reviewed and approved by the Texas Tech University Animal Care And Use Committee (Lubbock, TX, USA). Two-month-old zebrafish (*Brachydanio rerio*) were obtained from Aquatic Research Organisms (Hampton, NH, USA) and acclimatized for 4 weeks in zebrafish water (180 mg of Sea Salt® per liter of deionized water) in 10-gallon glass aquaria. Each tank was fitted with two hand-made internal biofilters consisting of 250-ml glass beakers filled with glass wool and glass beads. The water flow inside the filter was maintained by airflow through a glass pipette.

All physico-chemical water parameters were maintained at optimal conditions for zebrafish (temperature, 26-28 °C; dissolved oxygen > 4 ppm; pH, 6.5-8; photoperiod, 14:10-h light:dark, unionized ammonia < 0.1 ppm). Fish were fed twice daily to satiation with commercial diet (Tetramin®, Tetra Sales, Blacksburg, VA, USA). Dissolved oxygen, temperature, conductivity, and salinity were measured daily using a YSI® model 85 meter (Yellow Springs, OH, USA), and total ammonia was measured with a HACH® spectrophotometer model DR/2000 (Loveland, CO, USA) at least once a week. A small volume of water was siphoned from the bottom of the tank to remove debris once daily, and one-half of the water volume was removed and replaced with clean water once weekly.

14.2 Experimental Design and Perchlorate Exposure

Details of the experimental design and exposure protocols can be found in Study Protocol T9700/ZEB-02-01. Zebrafish were exposed to nominal AP concentrations of 0, 10, 100, 1000, and 10000 ppb. There were three replicate aquaria per treatment. Each experimental tank

contained approximately 50 fish at the beginning of the exposure. Fish were exposed to AP for 12 weeks and then were allowed to recover in the absence of perchlorate for an additional 12 weeks.

At the beginning of the exposure and at the time of each water exchange during the rearing period, the appropriate volume of AP stock solution was added to the aquaria to maintain the target perchlorate concentration. Water samples were collected for verification of actual perchlorate concentrations once every two weeks starting on the first day of exposure. At the end of the exposure period, the remaining fish were carefully removed with a net from the exposure tanks, briefly rinsed in respective large beakers with aerated clean zebrafish water, placed in clean 10-gallon glass aquaria with fresh zebrafish water, and reared for an additional 12 weeks. Water samples were collected during recovery period at 1, 4 and 12 weeks after transfer.

Fish for histological observations were collected at 2, 4, 8 and 12 weeks of exposure and at 4 and 12 weeks of recovery. At each sampling time, fish were allowed to swim ("rinsed") in three consecutive baths of fresh zebrafish water (to minimize contamination of the laboratory), euthanized in MS-222 (1 g/L), and placed in Bouin's fixative after an abdominal incision. Samples were also collected at 6 and 12 weeks of exposure and at 12 weeks of recovery for analysis of thyroid hormone concentrations in whole fish. For this purpose, fish were rinsed, euthanized, wrapped in aluminum foil, snap-frozen in liquid nitrogen, and stored at - 80 °C until further analysis. Five fish from each replicate aquarium, irrespective of sex, were randomly collected at each sampling. The sex of each fish was later verified by histology (except those for hormone analysis, which were frozen).

14.4 Perchlorate concentration in treatment water

Perchlorate concentrations in the stock solutions and in experimental tank water were verified by ion chromatography (Anderson and Wu 2002).

14.5 Growth

To evaluate the effect of AP on growth, body weight and fork length (from tip of snout to the point where the caudal fin bifurcate) were measured at each time of sampling. Condition factor was also calculated according to the formula, 100000 x body weight (g)/length (mm)^3.

14.6 Histology

Whole fish were kept in Bouin's solution for 48 h at 4 °C and subsequently processed for histology according to procedures described for zebrafish by Patiño et al. (2003). The head of the fish was separated by incision from the trunk region, and each piece was used to prepare separate blocks of paraffin for thyroid histopathology and sex determination, respectively. Sections (6 μm) were processed and stained with hematoxylin and eosin.

All thyroid histopathological analyses were conducted on the same cross-section of the head. This section was chosen according to its histological integrity and quality when viewing the first

row of sections on the slide from left to right. Digital images of thyroid follicles were taken with an Olympus (DP10) digital camera attached to Olympus microscope (BH2). Measurements were conducted digitally using Image-Pro® Express software (Media Cybernetics, Silver Spring, MD USA). The height of the thyroid follicle epithelium was used as index of hypertrophy. This height was measured at four predetermined positions (12, 3, 6, and 9 o'clock) in each of five follicles per fish. The average height was calculated for each follicle, and the average of the five follicles was determined for each fish. The number of blood vessels around the entire length of the follicular epithelium was counted under the microscope, and the perimeter length of the follicle was measured digitally. The number of blood vessels was standardized to 100 μm of follicular perimeter and used as index of angiogenesis for each follicle. Angiogenesis was determined in the same five follicles per fish, and the average of these determinations was regarded as the fish value. A semi-quantitative method was used to measure colloid depletion by assigning a score for each of the five follicles as follows: 0, no colloid depletion; 1, one-third of colloid depleted; 2, two-thirds of colloid depleted; and 3, no colloid present in the follicle. The average score for the five follicles was used as the fish value.

The procedures for fixation, processing and staining of the trunk regions were the same as for the head. The purpose of these preparations was simply to determine the sex of the fish by inspection of the gonads.

14.7 Thyroid hormone extraction and radioimmunoassay (RIA)

All five fish taken from each tank replicate at each sampling period were pooled for extraction. The total weight of the pooled fish was recorded, the fish were broken into smaller fragments while still frozen, and were then homogenized using a Tekmar® homogenizer in 3-4 volumes of ice-cold methanol (HPLC-grade) containing 1 mM propyl thiouracil (PTU). The homogenates were sonicated using a Fisher Scientific 50 Sonic Dismembrator at 3 x 20 sec pulses. Approximately 1000 counts per minute (CPM) of [^{125}I]-thyroxin (969Ci/mmol; Perkin-Elmer Life Sciences, Inc., Boston, MA, USA) in 50 μl of methanol (containing 1 mM PTU) were added to each homogenate, and the homogenates were incubated at room temperature for 30 minutes. [Immediately before use, free iodine was removed from the radiotracer preparation using Sep-Pak C_{18} cartridges (Waters) according to Denver (1993).]

Following the incubation, homogenates were centrifuged at 1000 *g* for 20 min at 4 °C. The supernatants were removed and mixed with 2 volumes of $CHCL_3$, and back-extracted into an aqueous phase with 1 to 3 ml of 2N NH_4OH followed by centrifugation at 1500 *g* for 15 min at 4 °C. The back extraction was repeated two more times. The aqueous fractions were pooled and dried in a Jouan centrifugal evaporator overnight. The dried samples were resuspended in 1 ml of 2N NH_4OH, mixed with 2ml $CHCL_3$, and centrifuged at 1500 *g* for 15 min at 4 °C. The aqueous phase was collected and purified by ion exchange chromatography as described by Morreale de Escobar et al. (1985). Briefly, Polyprep chromatography columns (Bio-Rad, Hercules, CA, USA) were prepared with 1.5 ml AG 1-x2 resin (200-400 mesh, chloride form, Bio-Rad) previously equilibrated with acetate buffer (pH 7). The columns were washed with 2 ml acetate buffer (pH 7) followed by a series of washes (2 ml each) including 100% ethanol, pH 4-acetate buffer, pH 3-acetate buffer, 1% acetic acid, and 35% acetic acid. Thyroid hormones

were eluted from the column by passing nine fractions (0.5 ml) of 75% acetic acid through the column. The first three fractions were discarded as they contained negligible amounts of [^{125} I] thyroxin. The remaining six fractions were pooled, evaporated, reconstituted in RIA buffer (300 µL), and the recovery of radiotracer was determined in an aliquot using a Cobra 5005 gamma counter (Packard, Downers Grove, IL, USA). Under these conditions, the recoveries of radiotracer ranged from 32-63 percent (average 44 percent).

The thyroxin content of reconstituted extract was determined by RIA following the procedure of Mackenzie et al. (1978). Briefly, duplicated 50-µL aliquots of each reconstituted extract were incubated in the presence of thyroxin rabbit antiserum (ICN Biomedicals, Inc., Costa Mesa, CA, USA) at a dilution of 1:1200, and [^{125}I] thyroxin (7000 CPM) for 90 min at 37 °C followed by a 16-h incubation at room temperature. The reaction was terminated by addition of goat anti-rabbit gamma globulin in ice-cold 5% polyethylene glycol followed by centrifugation at 4 °C. The radioactivity content of the pellet was determined with the gamma counter. Authentic thyroxin standards were run in parallel to the samples and in duplicate for each concentration. The hormone content of the samples was determined using a four parameter logistic transformation of [^{125}I] thyroxin displacement by the authentic standards. The values obtained for the two replicates per sample were averaged, and this average was corrected according to the estimated recovery for each sample. This RIA procedure was validated for zebrafish by performing parallel displacement curve analysis of serially diluted extracts and by determining the recovery of exogenously added authentic thyroxin into the extracts.

14.8 Data analysis

All analyses were conducted using Statistica® software package (StatSoft, Tulsa, OK, USA) at the level of significance $\alpha = 0.05$. The unit of replication for all treatments was the aquarium (fish tank). Thus, fish values within a tank were averaged to obtain tank values, and the sample size per treatment per sampling time thus was 3. However, because body size in zebrafish differs between sexes, the effects of AP on weight, fork length and condition factor were analyzed separately for males and females. Namely, a male and a female tank value were determined for each aquarium prior to the analysis. A preliminary analysis of hypertrophy, angiogenesis, and colloid depletion conducted on tank values calculated for each sex (3-way ANOVA; factors: sex, perchlorate concentration, and sampling time) indicated no significant differences between sexes. Thus, in the definitive analyses, all male and female fish values were pooled within a tank to obtain tank values for hypertrophy, angiogenesis and colloid depletion.

A tiered approach was followed for the data analysis. The first step was to conduct a 2-way ANOVA using perchlorate concentration (treatment) and sampling time as factors. If significant treatment or interaction effects were determined by this analysis, the second step was to perform a 1-way ANOVA for treatment means at each sampling time. Differences between means at each sampling time were determined using Duncan's multiple range tests.

15. Results

Fish tank #7 (100 ppb replicate) was lost during week 6 of exposure due to heater malfunction.

Thus, no data from this replicate was collected for weeks 8 and 12 of exposure or during the post-exposure period.

15.1 Perchlorate concentration in water

The measured concentrations of perchlorate in tank water were close to the nominal concentrations during the exposure period. The mean perchlorate concentrations (± standard error, n = 7 samples collected from each tank replicate) in the experimental tanks over the exposure period were 0 ± 0, 11 ± 0, 90 ± 3, 1131 ± 23 and 11480 ± 335 ppb respectively for the 0, 10, 100, 1000 and 10000 ppb nominal concentrations. During the recovery period perchlorate was detectable only once, in the first water sample taken (one week after transfer to clean tanks) from one of the tank replicates of the 100 ppb-treatment group - the concentration in this tank was 24 ppb. There were no traces of perchlorate detected at any other time or in any other tank during the recovery period. Although perchlorate levels during recovery were undetectable, for treatment identification purposes the results obtained during this period are associated with the original perchlorate concentrations in the appropriate results and discussion sections.

15.2 Animal growth and general behavior and appearance

The AP-treated zebrafish did not show any behavioral or physical signs of stress, and no treatment-related deaths occurred during the exposure or recovery period. The 3-month-old experimental fish were immature (juvenile stage) at the onset of the exposures, but had matured and showed signs of spawning behavior by the completion of the experiment 24 weeks later. Body weight, fork length and condition factor of male and female zebrafish were not affected by perchlorate exposure ($P > 0.05$), but there was an increase in the values of all three parameters during the experimental period (2-way ANOVA on each sex separately; $P < 0.05$). The overall weight, fork length and condition factor (mean of all perchlorate treatment groups ± SE) taken at 2 weeks of exposure were 0.21 ± 0.01 g, 26.8 ± 0.3 mm, and 1.06 ± 0.02 for males, and 0.26 ± 0.01 g, 27.5 ± 0.5 mm, and 1.23 ± 0.04 for females. The same measurements taken at 12 weeks of exposure were 0.51 ± 0.01 g, 38.4 ± 0.2 mm and 1.23 ± 0.01 for males and 0.55 ± 0.02 g, 33.0 ± 0.3 mm, and 1.40 ± 0.03 for females. Finally, at the end of the recovery period the measurements were 0.70 ± 0.01 g, 38.4 ± 0.2 mm, and 1.23 ±0.01 for males and 0.84 ± 0.02, 38.2 ± 0.3 mm, and 1.51 ± 0.02 for females.

15.3 Thyroid histopathology

Thyroid follicles normally are lined with a single layer of squamous or cuboidal epithelial cells and filled with colloid in their lumen (Fig.1A). Treatment with AP, especially at the higher concentrations and longer exposure times, induced the enlargement of the follicular layer (hypertrophy; Fig. 1B,C), an increase in the number of blood vessels within the follicular layer (Fig. 1B,C), and various degrees of depletion of colloid in individual follicles (Fig. 1C).

Quantitative analysis indicated that exposure to AP caused dose- ($P < 0.05$) and time-related ($P < 0.05$) responses in thyroid follicle hypertrophy, angiogenesis and colloid depletion (2-way ANOVA separately for each variable). At two weeks of exposure, the only variable for which

significant treatment effects were noted was angiogenesis, and the lowest observed effective level (LOEL) of measured perchlorate was 90 ppb (1-way ANOVA, $P < 0.05$; Fig. 2). At 4 weeks of exposure, hypertrophy became evident, and the LOEL for angiogenesis and hypertrophy were 1131 (1-way ANOVA, $P < 0.05$) and 11480 ppb (1-way ANOVA, $P < 0.05$), respectively (Fig. 2). At 8 weeks of exposure, the LOEL for hypertrophy remained at 11480 ppb (1-way ANOVA, $P < 0.05$) and for angiogenesis was 90 ppb (1-way ANOVA, $P < 0.05$) Fig. 2). At 12 weeks of exposure, the LOEL for hypertrophy had decreased to 1131 ppb (1-way ANOVA, $P < 0.05$) and for angiogenesis remained at 90 ppb (1-way ANOVA, $P < 0.05$) (Fig. 2). At 4 weeks of recovery, the LOEL for hypertrophy remained at 1131 ppb (1-way ANOVA, $P < 0.05$) and that for angiogenesis increased to 1131 ppb (1-way ANOVA, $P < 0.05$) (Fig. 3). At 12 weeks of recovery, hypertrophy was no longer detectable (1-way ANOVA, $P > 0.05$) but angiogenesis was still recognizable with a LOEL of 11480 ppb (1-way ANOVA, $P < 0.05$) (Fig. 3). It should also be noted that the absolute magnitudes of hypertrophy and angiogenesis in response to AP exposure tended to increase with time of exposure (Fig. 2) and to decrease with length of recovery (Fig. 3).

Signs of colloid depletion (mean score ± SE) were first noticeable after 8 weeks of exposure to perchlorate at 1131 ppb (0.04 ± 0.04) and 11480 ppb (0.24 ± 0.05), but only the latter was significantly different from the untreated control (1-way ANOVA and Duncan's multiple range test, $P < 0.05$). By 12 weeks of exposure, signs of colloid depletion were seen in the fish exposed to perchlorate at 1131 ppb (0.17 ± 0.13) and 11480 ppb (1.12 ± 0..20), and in both cases they were significantly different from the untreated control (1-way ANOVA and Duncan's multiple range test, $P < 0.05$). During the recovery phase, thyroid follicles regained their lost colloid relatively quickly. Although signs of colloid depletion were noticed at 4 weeks of recovery in fish exposed to 1131 ppb perchlorate (0.12 ± 0.10), this effect was not statistically significant (1-way ANOVA, $P > 0.05$). No signs of colloid depletion were observed after 12 weeks of recovery in any of the treatment groups.

15.4 Whole-body thyroxin content

No significant effects of AP exposure on whole-body thyroxin concentrations (ng/g body weight) were observed at any of the sampling times (2-way ANOVA; factors: treatment and sampling time; $P > 0.05$). The mean (± SE) concentration of thyroxine in fish tanks grouped by sampling time was 1.37 ± 0.10, 1.37 ± 0.09, and 1.73 ± 0.15 ng/g at 6 and 12 weeks exposure and after 12 weeks of recovery, respectively.

16. Discussion

This study evaluated six different biological endpoints in zebrafish as possible markers of perchlorate exposure: thyroid follicle angiogenesis, thyroid follicle hypertrophy, thyroid follicle colloid depletion, whole-body thyroxin concentration, body growth (weight and length), and condition factor. The results obtained indicated that histological indices of thyroid activity are, as a group, the most sensitive markers of AP-derived perchlorate exposure. In fact, zebrafish growth and condition factor as well as whole-body thyroxin concentration were not affected by perchlorate exposure in the present study.

The occurrence of angiogenesis, hypertrophy, hyperplasia, and colloid depletion in thyroid follicles of zebrafish exposed to AP has been previously reported (Patiño et al., 2003). However, the present study is the first to methodically examine the usefulness of these histological changes as markers of perchlorate exposure in zebrafish, and the first to examine angiogenesis as marker of perchlorate exposure in any species. Angiogenesis was by far the most sensitive of the histological markers examined on either temporal or perchlorate-concentration scales. Namely, angiogenesis appeared well before hypertrophy and colloid depletion, and was able to sense water concentrations of perchlorate at least one order of magnitude lower than those detected by the other parameters. Curiously, the LOEL of perchlorate for angiogenesis did not change with exposure time, essentially remaining at 90 ppb from 2 to 12 weeks of exposure. The absolute number of blood vessels per unit length of follicular perimeter, however, generally increased with time of exposure to the effective concentrations of perchlorate (90-11480 ppb). On a temporal scale, hypertrophy was next in order of sensitivity, becoming evident for the first time at 4 weeks but only at the highest concentration of perchlorate used (11480 ppb). Colloid depletion was not detected until after 8 weeks of exposure, also at 11480 ppb. The LOEL for hypertrophy and colloid depletion declined simultaneously from 11480 ppb to 1131 ppb at 12 weeks of exposure. Although these histological alterations were reversible after the removal of AP from tank water, significantly higher levels of thyroid follicle vascularization were still observed after 12 weeks of recovery from exposure to the highest concentration of perchlorate (11480 ppb). Thus, angiogenesis is also the most persistent of the markers examined in this study. Unlike the findings of Patiño et al. (2003), hyperplasia was not observed in the present study. This difference may be due to the higher concentrations of perchlorate (18000 ppb) used by Patiño et al. (2003), or to differences in sensitivity to perchlorate between the fish populations used by the two studies. Like the findings of Patiño et al. (2003), no sex-linked effects of AP were noted on the thyroid histology of fish in the present study.

The effects of perchlorate on thyroid function also have been examined in amphibians and other vertebrates. In *Xenopus laevis*, environmental concentrations of AP-derived perchlorate as low as 59 ppb caused hypertrophy of thyroid follicles after 10 weeks of exposure (Goleman et al., 2002a). This finding with amphibians contrasts with the results of the present study with zebrafish, where the lowest concentration of perchlorate that induced thyroid follicle hypertrophy after 12 weeks of exposure was 1131 ppb. In rats, oral administration of AP at 10 mg/kg/day caused significant increases in thyroid gland weight, follicular cell hypertrophy, micro-follicle formation, and colloid depletion within 14 to 90 days of exposure (Siglin et al., 2000). Like in zebrafish (present study), histological changes of the thyroid were reversible in *X. laevis* (Goleman et al., 2002a) and rats (Siglin et al., 2000) after a 28-30-day period of recovery in the absence of perchlorate.

Increased vascularization (angiogenesis) of the thyroid gland has been reported in rats orally treated with 1 percent potassium perchlorate after 2 months of treatment (Fernandez Rodriguez et al. 1991). Comparing the phenomenon of angiogenesis between zebrafish and rats is complicated because of differences in their thyroid structure. Thyroid follicles of zebrafish and other teleost fishes are not encapsulated in a discrete gland like they are in most other vertebrate taxa, but are found dispersed among the branchial arterioles of the lower throat region. The

increased vascularization observed in the present study seemed to occur inside the follicles within the follicular epithelium, but Patiño et al. (2003) also reported a general increase in the vascularization of the extrafollicular space around the thyroid follicles of zebrafish exposed to AP. In the rat thyroid gland, perchlorate-induced vascularization seems to occur in the extrafollicular compartment (Fernandez Rodriguez et al., 1991). Regardless of the microanatomical location of the angiogenic response to perchlorate, the increased vascularization can be regarded as a physiologically adaptive mechanism to compensate for disruptions in thyroid hormone production. Namely, an increase in the number of blood vessels in or around thyroid follicles may increase the supply of nutrients (including iodide) to the follicles, and also enhance the removal and distribution of thyroid hormones stored in their lumen in order to maintain thyroid hormone homeostasis.

Potassium perchlorate at a nominal concentration of 0.05 percent (approx. 50 ppm perchlorate) inhibited the uptake of radioactive iodide in the throat region of zebrafish larvae by 90 percent (Brown, 1997). Since iodide is necessary for thyroid hormone synthesis, curtailing its availability to the thyroid follicles inhibits the production of thyroid hormones and presumably also their secretion. Direct measurements of circulating or whole-body thyroid hormone concentrations, however, have yielded inconsistent results. For example, exposure to a nominal concentration of 0.01 percent potassium perchlorate (approx. 10 ppm) caused a reduction in serum thyroxin and triiodothyronine levels in sea lamprey (*Petromyzon marinus*) larvae after several weeks of treatment (Manzon and Youson, 1997). Also, AP-derived perchlorate at 14140 ppb, although not 59 ppb, caused a slight reduction in whole-body thyroxin concentrations of *X. laevis* tadpoles after 70 days of exposure (Goleman et al., 2002a). Decreased production of thyroxin, but not triiodothyronine or TSH, was observed in rabbits after about 3 weeks of oral administration of AP at ≥ 30 mg/kg/day (York et al., 2001a). Conversely, total thyroxin concentrations were significantly increased in deer mice after prolonged oral administration of 1 nM and 1 μM AP, whereas triiodothyronine concentrations were not affected (Thuett et al., 2002). Also, the results of the present study showed that whole-body thyroxin concentrations were not affected by exposure to perchlorate in zebrafish at concentrations as high as 11480 ppb. This variability in the effects of perchlorate on thyroid hormone levels among species may be explained by differences in the concentrations of perchlorate used in the various studies. Also, thyroid follicles can store significant amounts of hormone with the colloid, and species-specific differences may exist in the patterns and rates of depletion of these stores. The results of the present study showed that only about one-third of the colloid had been depleted in zebrafish exposed to perchlorate at 11480 ppb, even after 12 weeks of treatment. Overall, thyroid hormone concentrations, in the circulation or whole-body extracts, seem to be relatively insensitive and unreliable markers of perchlorate exposure.

Exposure to AP had no effect on the growth and condition factor of zebrafish. The fish were immature at the onset of the exposures but by the end of the experiment, 24 weeks later, they had grown in size and matured (as judged by displays of spawning behavior) irrespective of treatment. The time-dependent increase in condition factor of both males and females is likely due to the development of their gonads. Indeed, cursory inspection of gonadal sections prepared to determine the sex of the fish indicated that they reached reproductive maturity during the course of the study. It appears, therefore, that the general health and pubertal development of

zebrafish were not affected by AP exposure. The same conclusion was made by Patiño et al. (2003), who exposed adult (mature) zebrafish to AP-derived perchlorate at 18000 ppb for 8 weeks and found that general behavior and reproductive performance were unaffected despite drastic changes in thyroid histology. Similar results were obtained in a study with pregnant rabbits given AP orally, where fetal growth was unaffected at dosages as high as 100 mg/kg/day (York et al., 2001a). In *X. laevis*, however, AP in the ppb range inhibited the growth and metamorphosis of young larvae (Goleman et al., 2002b). Although perchlorate at very high concentrations (≥ ppm range) can affect the development of zebrafish embryos and larvae (Brown, 1997; Elsalini et al., 2003), the effects of environmentally relevant concentrations of perchlorate on larval fishes are unknown. More information is needed to determine if the growth and development of amphibians (larval or adults) are more susceptible to perchlorate exposure than those of fishes.

In conclusion, this study showed that angiogenesis is a much more sensitive marker of perchlorate exposure than hypertrophy, colloid depletion, whole-body thyroxin levels, body growth (weight and fork length), and condition factor. Namely, angiogenesis not only responded faster and at lower concentrations of perchlorate, but also persisted for longer periods of time after removal of perchlorate from the rearing water. Further, the LOEL for angiogenesis is within the environmentally relevant range of perchlorate concentrations.

17. Study Records and Archive

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18. References

Anderson TA, Wu TH. 2002. Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. *Bull Environ Contam Toxicol.* 68:684-691.

Brown DD. 1997. The role of thyroid hormone in zebrafish and axolotl development. *Proc Natl Acad Sci U S A.* 94:13011-13016.

Capen CC. 2001. Toxic response of endocrine system. *In Casarett and Doull's Toxicology: The basic Science of poisons.* Chapter 21,711-759.

Denver RJ 1993. Acceleration of anuran amphibian metamorphosis by corticotropin-releasing hormone-like peptides. Gen Comp Endocrinol 91:38-51.

Elsalini OA, von Gartzen J, Cramer M, Rohr KB. 2003.Zebrafish hhex, nk2.1a, and pax2.1 regulate thyroid growth and differentiation downstream of Nodal-dependent transcription factors. Dev Biol 263:67-80.

Fernandez Rodriguez A, Galera Davidson, H, Salguero Villadiego, M, Moreno Fernandez, A, Martin Lacave, I, Fernandez Sanz, J. 1991. Induction of thyroid proliferative changes in rats treated with antithyroid compound. Anat Histol Embryol 20:289-298.

Goleman WL, Carr JA, Anderson TA. 2002a. Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. *Environ Toxicol Chem.* 21: 90-597.

Goleman WL, Urquidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. 2002b. Environmentally relevant concentrations of ammonium perchlorate inhibit development

and metamorphosis in *Xenopus laevis*. *Environ Toxicol Chem* 21:424-430.

MacKenzie DS, Licht P, Papkoff H. 1978.Thyrotropin from amphibian (Rana catesbeiana) pituitaries and evidence for heterothyrotropic activity of bullfrog luteinizing hormone in reptiles. Gen Comp Endocrinol 36:566-574.

Manzon RG, Youson JH. 1997. The effects of exogenous thyroxin (T4) or triiodothyronine (T3), in the presence and absence of potassium perchlorate, on the incidence of metamorphosis and on serum T4 and T3 concentrations in larval sea lampreys (Petromyzon marinus L.). Gen Comp. Endocrinol 106:211-220.

Morreale de Escobar G, Pastor R, Obregon MJ, Escobar del Rey F. 1985. Effects of maternal hypothyroidism on the weight and thyroid hormone content of rat embryonic tissues, before and after onset of fetal thyroid function. Endocrinology 117:1890-1900.

Patiño R, Wainscott MR, Cruz-Li EI, Balakrishnan S, McMurry C, Blazer VS, Anderson TA. 2003. Effects of ammonium perchlorate on the reproductive performance and thyroid follicle histology of zebrafish. *Environ Toxicol Chem* 22:1115-1121.

Siglin JC, Mattie DR, Dodd DE, Hildebrandt PK, Baker WH. 2000. A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. *Toxicol Sci* 57:61-74.

Soldin OP, Braverman LE, Lamm SH. 2001. Perchlorate clinical pharmacology and human heath: a review. Therap Drug Monitoring 23:316-331.

Thuett KA, Roots EH, Mitchell LP, Gentles BA, Anderson T, Kendall RJ, Smith EE. 2002. Effects of in utero and lactational ammonium perchlorate exposure on thyroid gland histology and thyroid and sex hormones in developing deer mice (*Peromyscus maniculatus*) through postnatal day 21. J Toxicol Environ Health A 65:2119-2130.

Wolff J. 1998. Perchlorate and the thyroid gland. Pharmacol Rev 50:89-105.

York RG, Brown WR, Girard MF, Dollarhide JS. 2001a. Oral (drinking water) developmental toxicity study of ammonium perchlorate in New Zealand White rabbits. *Int J Toxicol* 20:199-205.

York RG, Brown WR, Girard MF, Dollarhide JS. 2001b. Two-generation reproduction study of ammonium perchlorate in drinking water in rats evaluates thyroid toxicity. *Int J Toxicol* 20:183-197.

19. Figures

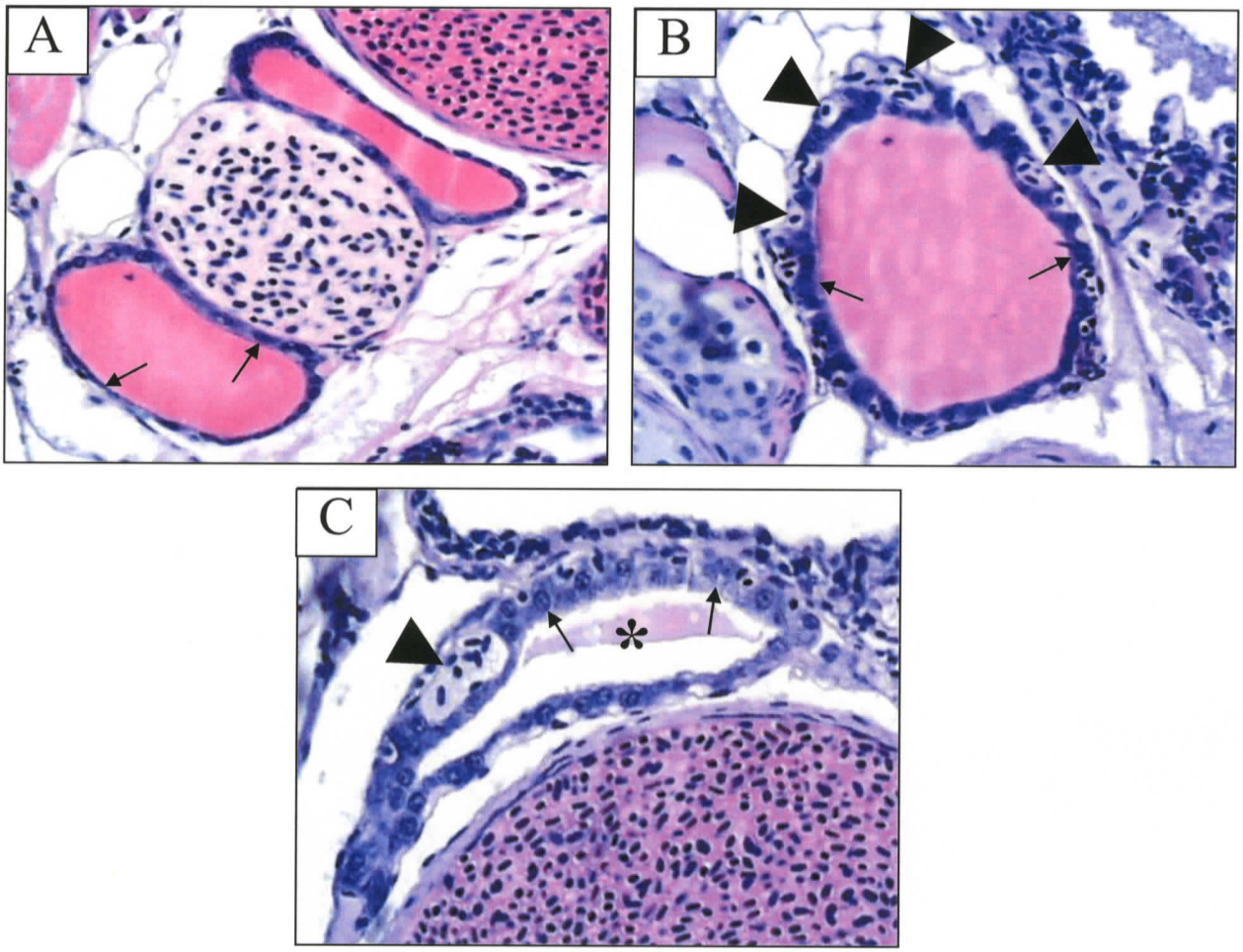

Figure 1. Photomicrographs of thyroid follicles of zebrafish reared in control water (A) or in water containing AP-derived perchlorate at measured concentrations of 11480 ppb for 12 weeks (B-C). Note the squamous epithelial cell layer in control fish (A, arrows), and the cuboidal-to-columnar shape of the cells in fish exposed to perchlorate (B-C, arrows). The follicles of perchlorate-exposed fish also contained higher numbers of blood vessels within the epithelial layer (B-C, arrowheads), and some follicles in these fish also showed signs of colloid depletion (C, asterisk). Hematoxylin-eosin stain. All photographs were taken at the same original magnification (x 100).

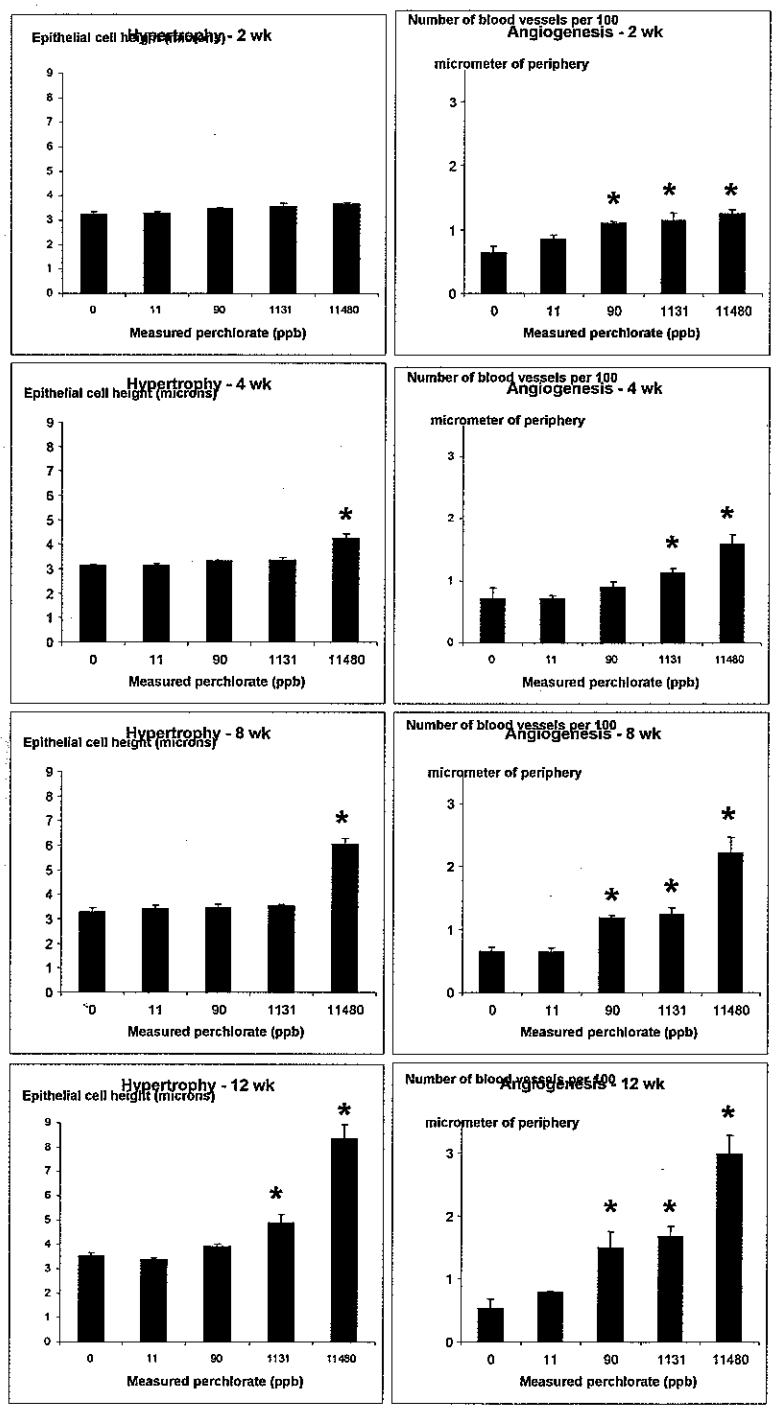

Figure 2. Changes in hypertrophy and angiogenesis of zebrafish thyroid follicles during exposure to AP-derived perchlorate at measured concentrations 0 to 11480 ppb from 2 to 12 weeks (wk). Bars associated with an asterisk are significantly different from the control (0 ppb) bar (Duncan's multiple range test, $P < 0.05$).

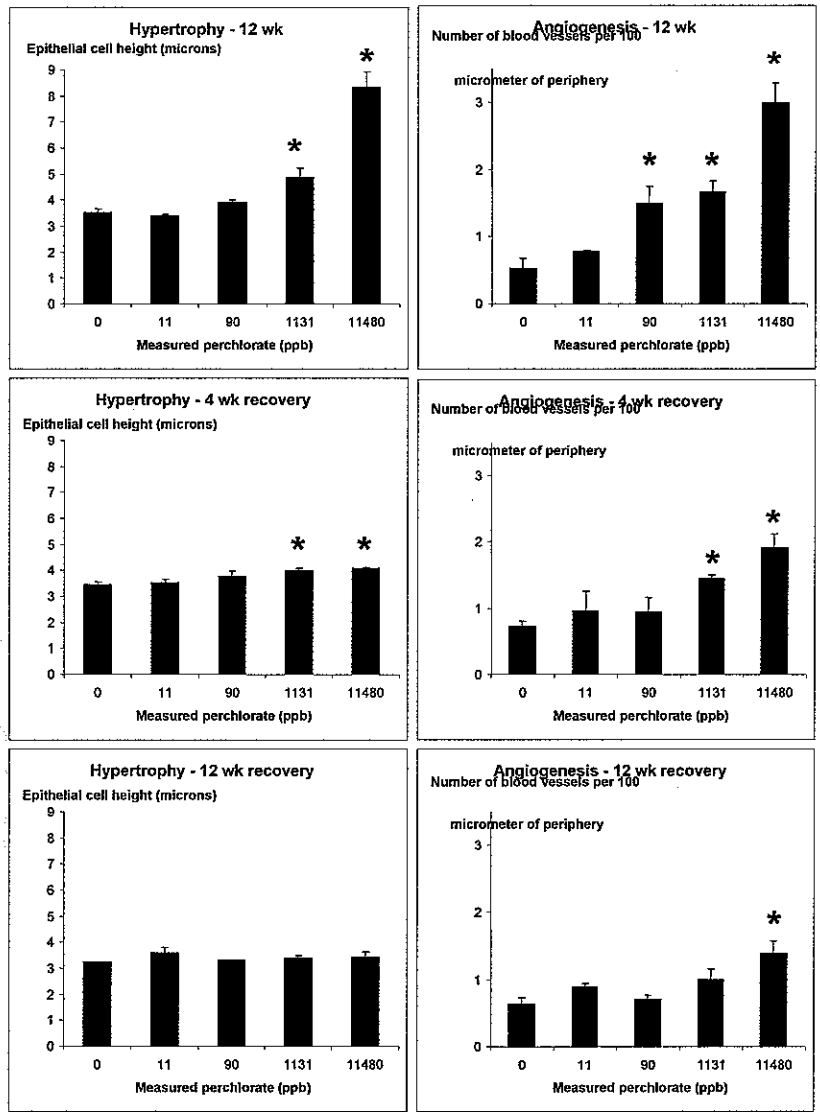

Figure 3. Changes in hypertrophy and angiogenesis of zebrafish thyroid follicles during recovery from exposure to AP-derived perchlorate at measured concentrations 0 to 11480 ppb for 12 weeks (wk; upper graphs). Measurements were taken after 4 (middle graphs) and 12 (lower graphs) weeks following removal of AP from the water. Bars associated with an asterisk are significantly different from the control (0 ppb) bar (Duncan's multiple range test, $P < 0.05$).

20. Appendices

Study Protocol
Changes to Study Documentation Forms

A STUDY PROTOCOL

ENTITLED

EFFECT OF AMMONIUM PERCHLORATE ON THYROID FUNCTION OF ZEBRAFISH

STUDY/PROTOCOL NUMBER:	ZEB-02-01
SPONSOR:	United States Air Force IERA/RSE 2513 Kennedy Circle Brooks Air Force Base, Texas 78235-5123
TESTING FACILITY:	Texas Coop Fish and Wildlife Research Unit - AP Texas Tech University Box 42120, 218 Agricultural Science Building Lubbock, Texas 79409-2120
TEST FACILITY MANAGEMENT:	Dr. Reynaldo Patiño
STUDY DIRECTOR:	Mr. Sandeep Mukhi
PROPOSED EXPERIMENTAL START DATE:	06-15-2002

1. DESCRIPTIVE STUDY TITLE

Effect of ammonium perchlorate on thyroid function of zebrafish

2. STUDY/PROTOCOL NUMBER ZEB-02-01

3. SPONSOR

United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME & ADDRESS

Texas Coop Fish and Wildlife Research Unit - AP
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES

Start Date: 06-15-2002
Termination Date: 12-31-2003

6. KEY PERSONNEL

Reynaldo Patiño, Testing Facility Management
Sandeep Mukhi, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator

7. DATED SIGNATURES

Reynaldo Patiño 7-8-02 Dr. Reynaldo Patiño
Testing Facility Management

S K Mukhi 07-08-02 Mr. Sandeep Mukhi
Study Director

7-22-02 Mr. Ryan Bounds
Quality Assurance Manager

Todd Anderson 7-22-02 Dr. Todd Anderson
Analytical Chemist

Ronald J Kendall 7/24/02 Dr. Ronald Kendall
Principal Investigator

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to Texas Tech University and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. Reynaldo Patiño
Texas Coop Fish and Wildlife Research Unit
Texas Tech University
Box 42120, 218 Agricultural Science Building
Lubbock, Texas 79409-2120

9. STUDY OBJECTIVES / PURPOSE

1. To determine dose-response and time-course of ammonium perchlorate effects on thyroid follicle cell hypertrophy and thyroid angiogenesis (histopathology)
2. To determine time-course of thyroid histopathological recovery following termination of ammonium perchlorate exposure.

10. TEST MATERIALS

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: white powder, specific gravity of 1.950.
Purity: Certificate of analysis will be obtained from the company. This chemical will be 99.999% pure as indicated by supplier.
Stability: incompatible with strong reducing agents, strong acids, heat-sensitive. The ammonia is a hazardous combustion or decomposition product.
Source: Aldrich Chemical Company

Reference Chemical name: Calcium Chloride
CAS number 10035-04-8
Characterization: coarse white powder or mixture with medium size granules.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Magnesium Sulfate
CAS number: 100-34-99-8
Characterization: colorless crystals
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Potassium Chloride
CAS number: 7447-40-7
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sea Salts
CAS number: Not applicable
Characterization: an artificial salt mixture closely resembling the composition of the dissolved salts of ocean water.
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Aquarium Systems, Inc.

Reference Chemical name: Sodium Bicarbonate
CAS number: 144-55-8
Characterization: white crystalline powder
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Sodium Chloride
CAS number: 7647-14-5
Characterization: white crystalline granules
Purity: certificate of analysis or analytical testing will indicate purity.
Stability: stable
Source: Sigma

Reference Chemical name: Ultrapure water with added salts needed by fish will be used as reference solution to which negative reference material or test material will be added for treatments.
CAS Number: Not applicable
Characterization: water quality will be tested by chemical analysis and pH will be monitored regularly.
Purity: ultrapure
Stability: stable
Source: City tap water or steam plant deionized water that has been run through a carbon filter and a de-ionizer to convert it to ultrapure water. Sea Salts (60-240 mg/L) will be added to this water, or a modified FETAX will be made.

11. JUSTIFICATION OF TEST SYSTEM

Ammonium perchlorate is known to prevent accumulation of iodine in thyroid follicles. Since iodine is necessary for the synthesis of thyroid hormones (T_3 & T_4), AP therefore lowers the production of thyroid hormones and thus has a goitrogenic effect via increased synthesis of thyroid stimulating hormone (Capen 2001). Such conditions caused thyroid hyperplasia in rat (Patel *et al.*, 1996), increased vascularization in thyroid glands of humans (Fenton *et al.*, 2000), and thyroid hypertrophy and inhibition of metamorphosis in South African frog, *Xenopus laevis* (Goleman *et al.*, 2002a, b). Ammonium perchlorate also caused the development of hyperplastic nodules, and the formation of small blood vessels (angiogenesis) in thyroid tissue of zebrafish (Patiño *et al* submitted).

In a study of the effects of AP with zebrafish, Patiño *et al.*, (submitted) confirmed that thyroid hypertrophy is a reliable marker of perchlorate exposure in this species (DoD/SRDP Project phase I/II). Angiogenesis seemed to be as sensitive to AP exposure as hypertrophy. Hypertrophy is caused by relatively rapid changes in the activity of thyroid follicle cells whereas angiogenesis involves changes in tissue vascularization patterns. Therefore, angiogenesis may require longer exposure times to develop and following removal of AP from water, longer times to recover than hypertrophy. This property of angiogenesis could make it an ideal biomarker for field exposures to perchlorate or other environmental goitrogens. Since sublethal field exposures to water-soluble contaminants (such as AP) are likely to be episodic with variable frequencies depending on discharges, rainfall, et cetera. We therefore propose to evaluate angiogenesis as a potentially sensitive and reliable biomarker of field exposure to perchlorate. Preliminary observations from field-caught amphibians (J.Carr, pers. comm) are consistent with the general applicability of angiogenesis as a biomarker of perchlorate exposure in the field. However, information on the time-responses and dose-responses of angiogenesis to AP is currently unavailable and it is needed to validate the utility of angiogenesis relative to other biomarkers such as thyroid follicle cell hypertrophy.

Zebrafish has been chosen as the experimental animal because they are a widely used animal model in basic toxicological, developmental, and reproductive research. Also they are easily and economically maintained. Moreover, this fish was used in our earlier experiments that generated the data concerning the effects of AP on fish thyroid histopathology.

12. TEST ANIMALS:

Species: Danio rerio, Zebrafish

Strain: Wild type

Age: Juveniles (over 1-2 month old) in zebrafish water (ultrapure water with 60-180 Mg/L Sea Salt) or modified FETAX (SOP AF-1-01).

Number: Approximately 700 Juveniles consisting of both male and female.

Source: We will use fish from Aquatic Research Organisms or other appropriate sources. Fish will be received by overnight mail and will be allowed at least one week to acclimatize (SOP AF-1-01) before their use in experiments.

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM

Zebrafish will be housed in single 10-gallon aquaria (filled with 28 liters of zebrafish water) containing approximately 50 fish per tank. Newly obtained fish will be allowed to acclimatize to their aquaria at least 1-2 weeks before exposure (same conditions as in SOP AF-1-01). Tanks will be appropriately labeled with a minimum of the following: project number, initials, concentration, chemical, replicate number, and date initiated. Treatment solutions will be added to the appropriate tanks according to SOP AF-1-01.

In this experiment we are proposing to do three separate analysis (tasks) but they will all be conducted simultaneously from the same experimental groups of fish. The various tasks to be conducted are described below.

Task–1 (Dose-response): Fish will be exposed to 0, 0.01, 0.1, 1 and 10 ppm of AP according to the SOP-AF-1-01.
Task –2 (Time-response): Samples for histological analysis will be taken at 2, 4, 8 and 12 weeks of exposure
Task –3 (Reversal effects after AP exposure termination): Samples for histological analysis will be taken at 2, 4, 8 and 12 weeks after exposure termination.

Fish will be fed frozen shrimp or other prepared diet twice daily. Feeding behavior and general animal health will be monitored daily. Water temperature, pH, salinity, dissolved oxygen will be recorded daily. One half of the water volume in the aquaria (14L) will be replaced twice weekly and ammonium level will be monitored once weekly. At each sampling time fish will be euthanized in a lethal concentration of anesthetic (1g/L MS-222) following the SOP AC-3-03. The samples will be fixed in Bouin's fixative, and further histological processing will be followed according to SOP AQ-2-10.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL

AP at 0, 0.01, 0.1, 1 and 10 ppm concentrations (or 5 treatments) will be placed into precleaned tanks (SOP AF-1-01) housing zebrafish. There will be 3 replicates per concentration of AP. At each sampling time, 5 fish will be sampled from each replicate. There will be a total of 8 sampling times including exposure and post exposure (see section 13). Therefore, total fish required = 5 dosages X 8 sampling times X 3 tank replicate X 5 fish per replicate = 600 fish. Each experimental tank will contain an additional number of 10 zebrafish to compensate normal mortalities during the experiment.

15. METHODS

15.1 Test System acquisition, quarantine, and acclimation.

Zebrafish will be obtained from Aquatic Research Organism or other appropriate sources. Fish will be maintained in zebrafish water (ultrapure water with sea salts or modified FETAX according to SOP AF-1-01) and under 14-light/10 dark cycle, 26-30^{0}C water temperature, pH 6.0-8.5. Fish will be maintained as indicated in the zebrafish AP Husbandry SOP AF-1-01.

15.2 Test Condition Establishment.

Conditions will be set according to zebrafish AP Husbandry SOP AF-1-01 (pH 6.0-8.5, 26-30^{0}C, 14/10 light/dark cycle), in ultrapure water with salts. Labeling will follow as described in Section 13. (For additional details on zebrafish husbandry see SOP AF-1-01). Fish will be placed into experimental tanks for at least 1-2 weeks prior to initiation of exposure. A concentrated solution of ammonium perchlorate will be added in the appropriate volume to the tanks to obtain the target concentrations. Tanks will be cleaned and maintained as indicated in SOP AF-1-01 for static renewal. Water removed will be replaced with the appropriate treatment water.

15.3 Test Material Application

Test material will be kept in stock solutions. To achieve the desired exposure concentrations (0, 0.01, 0.1, 1 or 10 ppm of AP), stock solutions will be mixed directly in the fish tanks with zebrafish water as indicated under SOP AF-1-01. Treatment solutions will be replaced every third or fourth day (twice a week) as indicated in greater detail in the static renewal procedures of SOP AF-1-01. The application will continue until the experiment is concluded.

Rates/concentrations: 0, 0.01, 0.1, 1 and 10 ppm

Frequency: Continuous exposure for the duration of the experiment, which will be no longer than 12 weeks. Sampling will continue after termination of exposure for an additional 12 weeks. During post exposure, the fish will no longer be exposed to the contaminant but they will be maintained according standard procedure (SOP AF-1-01).

Route/Method of Application: Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory as the chemical will be in the beaker/aquaria water.

Justification for Exposure Route: Exposure by rearing water is most appropriate as run off and sewage effluents enter water systems such as lakes and streams. Because fish respire, ingest, and are dermally exposed to the waters in which they live, this situation is most representative of field conditions.

Exposure Verification: A sample of each stock solution will be tested by chemical analysis by analytical chemistry manager.

15.4 Test System Observation

Histological sections will be randomly chosen from a slide and follicle cell height will be considered as index of hypertrophy. Increased blood vessel formation will be ranked positive or negative according to the presence or absence of more than five blood vessels whose respective diameter fit within a 50 x 50 μm^2 (Patiño *et al.*, submitted)

15.5 Animal Euthanization and Sample Collections

Fish (5 per replicate) will be euthanized at each sampling time with a 1 g/L buffered MS-222 solution (SOP AC-3-03). Subsequently the euthanized fish will be fixed in Bouin's fixative and histological procedure will be conducted according to SOPAQ-2-10.

15.6 Endpoint Analysis

Mortality of the experimental animals will be observed and recorded daily. Histological observation of thyroid gland will be our end point of observation. The hypertrophic condition and excess blood vessel development among different experimental treatment groups will be compared with that of the control group.

16. REPORT CONTENT/RECORDS TO BE MAINTAINED

Records to be maintained include room temperature, water temperature, dissolved oxygen, salinity, pH, conductivity, and ammonia concentrations.

Date, time, and number of feedings per tank will be recorded. Number of expired fish removed prior to termination of exposure will be recorded, including date, and tank number.

Report content will include presentation of data, interpretation, and discussion of the following

- Differences among treatments in thyroid blood vessel formation and nuclear hypertrophy
- Discussion of the relevance of findings
- List of all SOPs used
- List of all personnel

17. RECORDS TO BE MAINTAINED / LOCATION

The final report will be delivered to the Sponsor on or before 12-31-03. Copies of all data, documentation, records and protocol information shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

18. QUALITY ASSURANCE

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

19. PROTOCOL CHANGES / REVISIONS

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

20. REFERENCES

Capen, CC. 2001. Toxic responses of the endocrine system. In Klaassen CD, ed, Casarett and Doull's Toxicology: The basic Science of Poisons. McGraw-Hil, New Yrk, NY, USA, pp.711-759

Fenton C, Patel A, Dinauer C, Robie DK, Tuttle RM, Francis GL. (2000) The expression of vascular endothelial growth factor and the type 1 vascular endothelial growth factor receptor correlate with the size of papillary thyroid carcinoma in children and young adults. *Thyroid* 2000 Apr; 10(4): 349-57

Goleman WL, Carr JA, Anderson TA.(2002a) Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing Xenopus laevis. *Environ Toxicol Chem* 2002 Mar; 21(3): 590-7

Goleman WL, Urquidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. (2002b) Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis Environ Toxicol Chem* 2002 Feb; 21(2): 424-30

Patel VA, Hill DJ, Eggo MC, Beck s GP, Logan A., (1996) Changes in the immunohistochemical localization of fibroblast growth factor –2 transforming growth factor-beta 1 and thrombo spondin –1 are associated with early angiogenic events in the hyperplastic rat thyroid. *J. Endocrinol* 148 (3) 485-99

Patiño R, Wainscott MR, Cruz-Li EI, Balakrishnan S, McMurry C, Blazer VS, Anderson TA, Effects of ammonium perchlorate on the reproductive performance and thyroid condition of zebrafish (submitted)

Dept. of Biological Sciences (DBS)
Box 43131
Lubbock, TX 79409-3131
(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation __x__ Addendums

Document Reference Information
Check One: _x___ Protocol ____ SOP ____ Other _____
Title: Effect of ammonium perchlorate on thyroid function of zebrafish

Dated: _____08/16/02_____
Document # (if appropriate): __ZEB-02-01__
Page #(s): _____6_____
Section #: _____15.5_____
Text to reference: _____

Change in Document: In study protocol it was not mentioned to take fish samples before zebrafish were exposed to the ammonium perchlorate. On the starting date of experiment (07/25/02) we had collected 20 samples, out of which 10 numbers were fixed in Bouin's fixative and 10 were frozen in liquid nitrogen. These 20 numbers of sample were polled from the stock of zebrafish.
This is an addendum to the present study protocol.

Justification and Impact on Study: Our end point of observation of this current study protocol is to find the hypertrophy and angiogenesis of experimental animals. The samples collected before the start of experiments will be referred as one of the control groups and can be compared with the experimental animals at the end of experiment.

Submitted by: Signature: S.Mukherjee Date: 08/16/02

Authorized by: Study Director: S.Mukherjee Date: 08/16/02

Received by: Quality Assurance Unit: _____ Date: 8/16/02

* Sequentially numbered in order of the date that the change is effective

Dept. of Biological Sciences (DBS)
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(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: _x___ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: _x___ Protocol ____ SOP ____ Other _____
Title: Effect of ammonium perchlorate on thyroid function of zebrafish

Dated: _____08/16/02_____
Document # (if appropriate): __ZEB-02-01_____
Page #(s): _____5_____
Section #: _____13_____
Text to reference: __One half of the water volume in the aquaria (14L) will be replaced twice weekly.....

Change in Document: In study protocol it was mentioned to replace one half of the water volume twice weekly. Now we plan to change the half of the water of the aquaria once a week.

Justification and Impact on Study: Water exchange is done to maintain a good water quality and reduce the load of ammonia in the aquarium. We have observed that the water quality of the tank remains within the optimal range for zebrafish and the ammonia levels remains pretty below the acute level for the zebrafish. Therefore we decided to go for water exchange once in a week. This will also help us in reducing the volume of waste (ammonium perchlorate in water) generated from the experimental tanks.

Submitted by: Signature: SKMuke Date: 08/16/02

Authorized by: Study Director: SKMuke Date: 08/16/02

Received by: Quality Assurance Unit: _____ Date: 8-16-02

* Sequentially numbered in order of the date that the change is effective

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(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Effect of ammonium perchlorate on thyroid function of zebrafish
Dated: 11/02/02
Document # (if appropriate): Zeb-02-01
Page #(s): 5
Section #: 13
Text to reference: ____Task3 (Reversal effects after AP exposure termination): Samples for histological analysis will be taken at 2, 4, 8, and 12 weeks after exposure termination.

Change in Document: Samples for histological observation will be taken at 4 and 12 weeks after exposure termination.

Justification and Impact on Study: Due to unavailability of enough fish for four samplings after exposure is terminated, we propose to take fish samples at 4th and 12thweeks post exposure period.

Submitted by: Signature: SKMurali Date: 11/02/02

Authorized by: Study Director: SKMurali Date: 1-24-03

Received by: Quality Assurance Unit: Date: 1-14-03

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project #.T9700/ZEB-02-01
*Change No: 04
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Effect of ammonium perchlorate on thyroid function of zebrafish
Dated: 11/02/02
Document # (if appropriate): Zeb-02-01
Page #(s): 5
Section #: 13
Text to reference: ____One half of the water volume in aquaria (14L) will be replaced twice weekly and ammonium level will be monitored once weekly.

Change in Document: Ammonia level was monitored more than once in a week for better management of water quality. Water was still changed once a week

---

Justification and Impact on Study: Ammonium level in the tank depends upon the pH of the water body. Daily fluctuation of pH causes a wide variation of ammonium concentration. Ammonia is highly harmful to the living organism (fish in our experiment). So we planed to monitor ammonia in the experimental tank more than once, (mostly twice in a week).

Submitted by: Signature: _____ Date: 11/02/02

Authorized by: Study Director: _____ Date: 1-21-03

Received by: Quality Assurance Unit: _____ Date: 1-21-03

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

29 MAR 2004

Perchlorate-Induced Alterations in Metabolic Rate and Thermoregulation in Prairie Voles (*Microtus ochragaster)* and House Sparrows (*Passer domesticus*)

STUDY NUMBER:	MRT-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	April 2003
RESEARCH COMPLETION:	December 2004

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List of Figures and Tables

Figure 1. Weekly mean resting metabolic rates (ml g^{-1} h^{-1}) of prairie voles exposed to perchlorate in the drinking water (n=21).

Figure 2. Peak metabolic rates (ml g^{-1} h-1) of prairie voles exposed to perchlorate for 51 days. Temperature in metabolic chamber held at 12°C for 20 minutes followed by a drop in temperature to 5°C for a maximum duration of 60 minutes.

Figure 3. Weekly mean resting metabolic rates of house sparrows dosed with perchlorate through 21 days of dosing. Experiment terminated after 21 days of dosing due to the high occurrence of disease in the sparrow colony, thus compromising the results of the study.

Table 1. Mean prairie vole plasma thyroxine (ug/dL), triiodothyronine (ng/dL), and liver and kidney weights (g/g b.w.) following 51 days of exposure to perchlorate in the drinking water (n=20).

Table 2. Individual and mean house sparrow thyroxine concentrations (ug/dL) and liver and kidney masses (g) following 3 weeks of perchlorate dosing.

Table 3. Mean concentrations (ug/g d.w.) of perchlorate in house sparrow livers and kidneys following 3 weeks of perchlorate dosing.

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Philip N. Smith

3/25/04
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 25, 2004
Date

1.0 **DESCRIPTIVE STUDY TITLE**: Perchlorate-Induced Alterations in Metabolic Rate and Thermoregulation in Prairie Voles (*Microtus ochragaster)* and House Sparrows (*Passer domesticus*)

2.0 **STUDY NUMBER**: MRT-03-01

3.0 **SPONSOR:**
Strategic Environmental and Research
Development Program
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4.0 **TESTING FACILITY**: The Institute of Environmental and Human Health
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5.0 **PROPOSED EXPERIMENTAL START & TERMINATION DATES**:
Start Date: April 1, 2003
Termination Date: December 31, 2003

6.0 **KEY PERSONNEL**:
Dr. Philip N. Smith, Study Director / Study Advisor
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Tina Kaviani, lab assistant
Lisa Perlmutter, research technician
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
- To evaluate the effects of perchlorate on metabolic rate and thyroid function in prairie voles and house sparrows through analysis of thyroid hormones, tissue perchlorate concentrations, and comparison of volume of oxygen

consumed (VO2) between treatment and control groups. Previous studies have shown elevated metabolic rates in thyroid hormone-administered birds and rodents (Banta and Homlcombe, 2002; Burger and Denver, 2002); depressed metabolic rates and circulating thyroid hormone concentrations coincide with hypothyroidism, which are often effects of perchlorate exposure

- To evaluate thermoregulatory costs associated with perchlorate exposure in prairie voles and house sparrows by measuring metabolic rate during cold stress experiments. Thyroid hormones are the key controllers of metabolism that are necessary for maintenance of constant body temperature in homeothermic animals; disruptions of shivering or non-shivering thermogenesis in birds or rodents, respectively, may place them at a disadvantage when faced with highly variable temperature extremes.

8.0 TEST MATERIALS:

Test Chemical name: ammonium perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure
Source: Sigma Aldrich

9.0 JUSTIFICATION OF TEST SYSTEM:

The prairie vole is a ground-burrowing rodent found in the north and central plains of the United States and in southern Canada (U.S.EPA, 1993). Voles are largely herbivorous, consuming primarily green succulent vegetation, but also roots, bark, and seeds as well. These small, non-hibernating mammals living in the north temperate and boreal regions are faced with seasonal changes in their thermal environment. They must be able to adapt to both hot and cold extremes, otherwise, they may be at a disadvantage when stressed with a contrasting season or extreme daily temperature fluctuation. Animals need enough insulative and/or thermogenic capability to withstand low temperature exposure without becoming hypothermic, and they must cope with cold stress by behavioral avoidance and/or increased thermogenic capacity (Wunder et al., 1977). Small mammals have a limited ability to increase their insulation, therefore behavioral avoidance and thermogenesis become the most important means for maintaining a relatively narrow range of body temperature (Haim and Izhaki, 1993).

Perchlorate is known to inhibit the uptake of iodide by the thyroid. This limitation of iodide availability may result in decreased production of thyroid hormones. Deficiencies in thyroid hormones have been associated with slow heart rate and increased sensitivity to cold (Danforth and Burger, 1984). Therefore, perchlorate-induced alterations in thyroid hormones may have secondary effects on metabolic rate and thermoregulation.

The house sparrow is a common passerine species that is often used as an avian behavioral and toxicological model. Birds, especially passerine species, are often

used as sentinel species when assessing the impacts of chemicals on ecosystems and populations. The insulative needs of birds are often met by a combination of plumage characteristics, behavioral avoidance of cold exposure, and shivering thermogenesis (Marsh and Dawson, 1989). Subcutaneous fat tends to be more localized in birds than in mammals, therefore birds cannot rely on fat storage for insulation. Incubation in females, as well as males, can also potentially expose the incubating parent to extremely demanding thermal conditions (Marsh and Dawson, 1989). Altered thyroid hormone concentrations influence the overall metabolic energy supply, disrupt liver glycogen storage, and decrease plasma glucose levels (Danforth and Burger, 1984). Cold temperatures tend to increase thyroid hormone secretion rates, and decreases in iodide transport into the thyroid gland from the blood tend to decrease thyroid hormone secretion rates (McNabb, 1995). Therefore, an association may exist between perchlorate reductions in thyroid hormone and decreased metabolic rates in animals.

Available toxicity data suggests that rodents may be sensitive to low levels of perchlorate in the environment (Thuett et al. 2002; Siglin et al. 2000). Past research has shown that perchlorate administration at similar concentrations increased thyroid weights, decreased thyroid hormone levels, and increased thyroid follicular hyperplasia (Siglin et al. 2000). McNabb et al. (in press) has noted perchlorate-induced alterations in circulating thyroid hormones and thyroid gland hormone of adult and young northern Bobwhite quail. Perchlorate has been detected at high concentrations in water, vascular plant tissues, and seeds at concentrations ranging from approximately 1 ppm to as high as 5000 ppm (Smith et al., 2001; data on file, TIEHH). Small mammal species and passerine birds may be found in such contaminated environments; therefore such species represent potentially useful models for assessing the ecological impacts of perchlorate exposure.

10.0 TEST ANIMALS:

Species: Prairie voles (*Microtus ochragaster)* and House sparrow (*Passer domesticus*)
Strain: Prairie voles (laboratory); House Sparrows (free-living)
Age: voles (21 - >100 days); sparrows (hatching year or after hatching year)
Number: approximately: voles (51); birds (50)
Source: voles (Texas Tech University vole breeding colony); birds (captured from Lubbock, TX)

11.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All voles were placed in standard rodent cages and each cage was labeled with a note card containing the appropriate identification information for the animal. Birds were placed into individual labeled cages containing the appropriate identification information for the animal on the front of the cage. All birds were color banded, with a unique color code being assigned for each bird. Collected

samples were placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

12.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Oxygen consumption was determined for voles that were exposed to perchlorate in drinking water and house sparrows dosed with perchlorate in water, as well as for control voles and sparrows. Metabolic rates were determined for animals in a resting state and cold-stress conditions. Metabolic rates of perchlorate exposed and non-exposed voles and dosed and non-dosed birds were compared to detect for physiological responses. Concentrations of thyroid gland hormones were examined to evaluate for thyroid hormone imbalance and dose-related responses. Vole and sparrow tissue samples were also analyzed for perchlorate. All voles and birds were randomly assigned into exposure groups (0, 1.0, and 10.0 mg/kg) and dosing groups (0, 12.5, and 25.0 mg/kg), respectively.

13.0 METHODS:

Experimental Groups

Twenty-one male voles were acquired from a breeding colony located at Texas Tech University. All voles were housed in acrylic Nalgene rodent cages (25 cm long, 15 cm wide, 11 cm high) with Aspen shavings. Purina laboratory chow and water were provided ad libitum. Voles were maintained in a temperature controlled room at 25-26°C and 30-40% relative humidity on a 12h:12h light:dark photoperiod. All animals were weighed daily between 1200 and 1500 hours and food and water consumption were monitored on a daily basis during pre-exposure and exposure periods. Voles were provided access to water of one of three target exposures: control (milli-Q water; n=7), low exposure (1.0 mg perchlorate/kg; n=7), or high dose (10.0 mg perchlorate/kg; n=7).

Adult male house sparrows (*Passer domesticus*) were captured in mist-nets or funnel traps from residential areas in Lubbock, Lubbock County, Texas (Scientific Collecting Permit: SPR-1098-984) during September and October 2003. Following capture, birds were transported to the laboratory and assessed for general health. Any birds showing signs of disease or injury were not used in the study. Sparrows were held indoors in separate cages (40 cm x 40 cm x 40 cm) under controlled temperature (25°C), light (12L:12D) and humidity (30-40% relative humidity) before and during all experiments. Birds were provided with food (equal mixture of white millet, Purina Mazuri small bird maintenance, and black oil sunflower seed), water, and mineral grit for the duration of captivity. All sparrows were maintained in captivity for approximately 2 weeks before experimentation. For perchlorate dosing, sparrows were divided into three treatment groups, control (n=7), low dose (12.5 mg perchlorate/kg; n=7), and high dose (25.0 mg perchlorate/kg; n=7). The dosing solution was administered with a

pipette by placing the tip of the pipette on the edge of the lower mandible and allowing the bird to drink 100 µL of the dosing solution. Sparrows were dosed once daily between the hours of 1200 and 1500.

Resting Metabolism

The metabolic rate of an inactive vole measured during the rest phase of its daily cycle was designated as the resting metabolic rate. All animals were allowed free access to food and water prior to metabolic experiments. Resting metabolic rate (RMR) was the lowest rate of oxygen consumption averaged over a continuous 5-minute period at 28°C (within the thermoneutral zone for this species) measured between 07:30 and 15:00 hours.

For all RMR measurements, voles were weighed and then placed in gas-tight metabolic chambers (modified plastic rodent cages; volume 2.3 L). Chambers were fitted for inlet and outlet tubes. In our system, gas-cylinder air (dry grade; 20.95% oxygen) was pumped into a climate-controlled chamber, where it was then channeled through a mass flow controller and a gas multiplexer (G245 and G244, respectively; Qubit Systems, Ontario, Canada). Gas then flowed into the chambers and excurrent air was rendered dry by passing it through a dessicant-filled column (magnesium perchlorate). A subsample of air (60 mL min^{-}1) from the excurrent air stream and was rendered carbon dioxide free by passing through a soda lime column, and then routed into a differential oxygen analyzer (DOX) (S104; Qubit). The airline entering into the DOX was split to where half of the sample air was pulled through a carbon dioxide analyzer (S154; Qubit) so that the sample reached the analyzers simultaneously. Metered air was directed to a single respirometry chamber for measurement, while simultaneously flushing air through 3 other unmeasured chambers. Air flow rates were adjusted to 850 mL min^{-1}(standard temperature and pressure conditions) for all vole chambers. During each trial, the automated respirometry system was programmed to measure oxygen consumption and carbon dioxide production for each vole at 1 second intervals for 15 minutes per chamber then switch to the next chamber in series. All data were collected by Labview 6.0 (National Instruments, Austin, TX) and were imported into separate Microsoft Excel files for each gas channel. Before beginning measurements on the next vole in series, we allowed for a 15 minute flush to ensure that residual gases had been removed from the system. Gas concentrations were measured in an empty chamber to obtain baseline levels passing through experimental chambers. The number of animals that could be run simultaneously (three per day) was due to behavioral characteristics (not fully diurnal or noctural) of the voles, and thus limited the sample sizes at each metabolic rate sampling period. Behavioral criteria and a video camera inside the temperature cabinet were used to determine whether animals were inactive during measurment of oxygen consumption. At the conclusion of each run, all animals were placed back into assigned cages. This type of format continued for 7 weeks for perchlorate experiments and 4 weeks for thyroxine experiments. Each animal was tested once per week.

Bird resting metabolic rate was measured during the night (20:30-06:30), and RMR calculated as the lowest rate of oxygen consumption averaged over a continuous 5-minute period at 30°C (within thermoneutrality; Hudson and Kimzey, 1966). The basic methods of collecting resting metabolic rates are as stated previously, with a few exceptions. Birds were weighed and the beginning of the dark cycle and placed into metabolic chambers fashioned from 3.8 L paint cans fitted with a perch, inlet and outlet, and sealed with paraffin wax. Flow rates were set at 850-900 mL min^{-1}(standard temperature and pressure conditions). Birds were allowed to acclimate in the chamber approximately 45 minutes prior to testing, and excurrent oxygen concentration was then measured at 1 second intervals for a 20 minute time period throughout the night. Three birds were tested per night and all birds were weighed and returned to cages following metabolic measurements.

Peak Metabolic Rate (cold-stress)

Maximal oxygen consumption during cold stress was determined using a respiratory gas mixture of approximately 80% helium and 20% oxygen (helox; Rosenmann and Morrison, 1974). For sliding cold exposure tests, individual animals were exposed to a series of declining temperatures in helox. Cold stress temperatures were held at 12°C for 20 minutes followed by a continuous drop in temperature (~0.5°C per minute) until reaching 5°C. Helox tests were conducted for 1 hour or until the vole became hypothermic (indicated by a steady decline in oxygen consumption over several minutes). Cold stress tests were conducted between 1200 and 1600 h on voles which were allowed free access to food and water prior to metabolic tests. Flow rates were maintained at 1000-1050 mL min^{-1} for measurements of peak metabolic rate. The mass flow monitor was calibrated for helox gas with the use of a wet cell calibrator (Gilian Gilibrator 2; Sensidyne; Clearwater, FL). Voles were weighed to the nearest 0.01 grams before and after testing with a electronic balance. Metabolic chambers (volume 850 mL) consisted of a borosilicate glass tube capped on both ends fitted with an inlet or outlet. One end of the tube contained an electrical fan (velocity of 6.3 ft^3 min^{-1}) that aided in circulating air inside the metabolic chamber. One animal from each group (control, low dose, or high dose) was tested at a time, instead of three at a time for this procedure. Before animals were tested, all chambers (2 blanks and 1 animal) were flushed with helox for at least 5 minutes at flow rates of 1000-1050mL min^{-1}. Colonic temperature was taken before cold exposure and within 60 seconds after being taken out of the chamber. Peak metabolic rates were calculated as the average of the highest rate of oxygen consumption over a 2-min period.

Animal Sacrifice and Sample Collections

Animals were weighed and then anesthetized in a saturated carbon dioxide chamber. Blood samples were then collected from each vole and bird, placed into

heparinized microcentrifuge tubes, and centrifuged until plasma had been separated. Plasma was then transferred to labeled tubes and frozen at -80°C until analysis. Following blood sampling, all animals were euthanized and necropsied. Livers, kidneys, and thyroid glands were collected and frozen until further analysis.

Hormone Analysis

Vole plasma total thyroxine (T_4) and total triiodothyronine (T_3) were measured using clinical radioimmunoassay (RIA) kits (Diagnostic Products Coat-A-Count; TKT4X and TKT3X, respectively). The assay procedures for plasma that accompaned the kits were followed, except for the inclusion of a additional calibration points. Calibration points for the thyroxine standard curve were 0, 0.2, 0.4, 0.6, 1, 4, 10, 16, 24 µg/dL. Plasma samples (35 uL) were analyzed in duplicate. Calibration points for the triiodothyronine standard curve were 0, 5, 10, 15, 20, 50, 100, 200, and 600 ng/dL. Plasma samples (125 uL) were analyzed in duplicate. Both total thyroxine and triiodothyronine radioimmunoassay kits were validated for prairie vole plasma by testing various volumes of plasma against the standard curve for parallelism, and spiking plasma samples of known thyroxine concentration with standards from the RIA kit.

Sparrow total triiodothyronine was not analyzed because there was not enough plasma to allow for an adequate sample size. Sparrows were analyzed using the RIA kits described above for total thyroxine, however several of the 21 birds had died before plasma samples were collected, therefore not allowing statistical analysis on sparrow thyroxine concentrations. Calibration points for the T4 standard curve were 0, 0.2, .05, 1.0, 4.0, and 10.0 ng/dL. Plasma samples (100 uL) were analyzed in duplicate and the total thyroxine radioimmunoassay kit was validated for house sparrow plasma.

Tissue Perchlorate Analysis

Ion chromatography was used to analyze vole and sparrow liver and kidney tissues for perchlorate. Tissue samples were allowed to thaw, and a wet weight for each was recorded. Samples were allowed to air-dry for approximately 48 hours and reweighed. Perchlorate was extracted from the samples (entire tissue) using an Accelerated Solvent Extractor (ASE; ASE 200, Dionex Corporation). The following operating conditions were used: Milli-Q water as extraction solvent, 1500 psi, 1 cycle, 60% flush, 5 minute preheat, 5 minute static, and 100°C oven. Total extraction time was 15 minutes per sample. Total volume of extract collected (~22 ml) was measured and recorded. A 1:10 dilution of each sample extract was prepared. Samples were then cleaned using silica and C18 solid phase extraction (SPE) cartridges. Extracts were then filtered through 0.45 µm Acrodisc® filters (Pall Gellman, Ann Arbor, MI). Tissue samples were analyzed via a preconcentration / preelution ion chromatography method (Tian et al., 2003). Samples were concentrated on a Dionex TAC-LP1 with 10 mM

sodium hydroxide eluent for 2.5 minutes and then injected into the separation system. Ion separation occurred on an analytical column (Dionex IonPac AS16) and using 100 mM sodium hydroxide. Total run time was 12.5 minutes with a 0.92 mL/min flow rate and 1000 µL injection volume. Retention times were used to identify perchlorate and the peak area was used for quantification. A standard curve was generated from calibration standards of 4, 50, 100, 150, and 300 ppb to determine sample concentrations.

Statistical Methods

Effects of exposure on liver peak metabolic rates and thyroid hormone concentrations were tested using analysis of variance. Any significant interactions were analyzed using Tukey's test. The effect of dosage on oxygen consumption rates, body mass, food consumption (g consumed/g b.w.), and water consumption (g consumed/g b.w.) over time were tested with repeated measures analysis of variance using the general linear model procedure in SAS. Pearson's correlation test was used to assess potential relationships between plasma thyroxine and peak metabolic rate in voles. Statistical tests were considered significant when $p \leq 0.05$. There were no deviations from the assumptions of normality or equality of variances for any of the analyses.

14.0 RESULTS

Voles:

Perchlorate effects on Metabolism

There were no significant differences in resting metabolic rate between any of the treatment groups during pre-exposure measurements (p=0.6049). There was no treatment effect on resting metabolic rate during any of the 6 weeks of exposure (Figure 1). The behavior nature of the voles may have obscured differences (see conlusions). There were also no significant differences in peak metabolic rate between any of the treatment groups on exposure day 51 (p=0.7985). High variability if peak metabolic rate between animals of the same treatment and low sample sizes may have obscured differences (Figure 2). Circulating T4 levels were not significantly correlated with peak metabolic rate (p=0.1464, r^2=0.1199).

Thyroid hormones

Mean thyroxine concentrations from perchlorate exposed voles decreased in a dose-dependent manner, however there were no significant differences between any of the perchlorate treatment groups (n=21, p=0.0871). Plasma triiodothyronine (T3) levels did not differ between any of the treatment groups following 51 days of exposure (p=0.6782). The smaller sample size (n=15) for T3 analysis probably explains why there is such a large difference between p-values for T3 and T4 analyses. Plasma volumes were not adequate for both T4

and T3 analysis for some voles. Plasma thyroid hormone levels were not analyzed for voles implanted with thyroxine-releasing pellets.

Other Physiological Indices

Mean body mass did not differ among treatment groups for any of the monitored weeks, pre-exposure or post-exposure. While food consumption did not vary significantly among treatment groups for any of the weeks monitored, water consumption was significantly higher for the 10.0 mg/kg treatment than the control group during the pre-exposure week and weeks 2,3,4,5, and 6 post-exposure. Mean water consumption differences between the 10.0 mg/kg and control groups remained relatively constant from the pre-exposure period through post-exposure weeks, therefore any differences between these groups were not likely attributable to perchlorate exposure.

Tissue Concentrations
Prairie vole livers and kidneys contained no detectable levels of perchlorate. This result is fairly consistent with other laboratory rodent studies that have been conducted at Texas Tech University (data on file, TIEHH).

House Sparrows:

Following acclimation, 21 out of 24 sparrows were selected to take part in experiments assessing perchlorate effects on metabolic rate. Following weeks 2 and 3 of perchlorate exposure, several birds had become ill and expressed symptoms of a bacterial infection (*Mycoplasma gallisepticum*), resulting in House Finch Disease, also referred to as Chronic Respiratory Disease. Most birds with the illness would die within 2-3 days after showing first symptoms. Eight of twenty-one sparrows became infected with the bacteria. Birds with the disease had as high as two-times the metabolic rate of uninfected birds. The results of the study were compromised due to this epidemic and the study was terminated after the 5th exposure day of the 3rd week. The room, cages, and all supplies located in the room where sparrows were located were decontaminated and a completely new set of birds was brought into the animal facilities center. This time, all birds were placed on the recommended mg/kg dosage of Tylan antibiotic (soluble-powder) for the recommended dosing period. All birds were monitored for illness during this period. Two weeks after treatment with Tylan, 4 more birds showed symptoms of House Finch Disease and died within 2 days of first symptoms. At this point, it was determined that this species was not going to be a good test species for this study, and it was too late in the study to redo the experiment. Interestingly, no sparrows showed symptoms of the respiratory disease prior to perchlorate dosing experiments (mid-Fall, 2003).

Circulating thyroxine levels and liver and kidney weights are shown below (Table 2). The mean perchlorate concentration detected in livers of house sparrows dosed with 12.5 mg/kg of perchlorate was 57.41 ± 68.77 ug/g (d.w.)

(Table 3). The mean perchlorate concentrations detected in kidneys from this group were 34.85 ± 42.83 ug/g (d.w.). Mean perchlorate concentrations detected in the liver and kidneys of the 25.0 mg/kg treatment group were 234.48 ± 258.26 ug/g (d.w.) and 362.98 ± 337.18 ug/g (d.w.), respectively. Metabolic rates of perchlorate-dosed birds through day 21 are shown below as well (Figure 3). Statistical analyses were not performed on any of the sparrow data due to the confounding factor of disease. In general, birds with the respiratory disease had increased liver and kidney wet weights. Another significant finding was that 4 of the 6 perchlorate-dosed sparrows had plasma T4 levels below detection limit (0.067 ng/dL), while all of the control animals analyzed had detectable T4 levels (Table 2).

15.0 DISCUSSION

The lack of a detectable energetic cost associated with perchlorate exposure in prairie voles is evidenced by relatively unchanged resting metabolic rates in exposed voles. If there were added costs, the sensitivity of our methodology or instrumentation did not allow for detection, or perchlorate exposure had no effect on the amount of energy allocated for maintenance costs. Added costs could have been compensated by the hypothalamus-pituitary-thyroid (HPT) axis or perhaps the exposure dose was not high enough or exposure duration not long enough for there to be an impact on metabolic function. To our knowledge, there have been no other studies investigating perchlorate effects on metabolic rate in endothermic organisms; however other endocrine disruptors acting on the thyroid gland have yielded results similar to our own (French et al., 2001). The indication of no difference in RMR among treatments groups and the large variability among weeks within a treatment group may have been attributable to an increase in activity within the metabolic chamber. In some cases, test voles may have rested a total of 30 minutes in the metabolic chamber per 7-8 hour test period. The configuration of the respirometry system and confounding factors such as long wash times (time taken for recirculation of gas in a chamber) made the process of collecting metabolic rates relatively difficult. Most voles expressed circadian rhythms when not taking part in metabolic experiments, however, these rhythms seemed to be disrupted during testing periods. Some voles may not have reached a true resting state, therefore not yielding a true resting metabolic rate. Other species, such as some passerine birds or deer mice, may be a better model to use when investigating contaminant effects on metabolic function. Animals that are more diurnal or nocturnal (e.g. deer mice) usually produce much more stable resting metabolic rates and allow for more than one stable reading per test period (data on file, TIEHH).

Initially we expected to see an increase in metabolic rate in the two exposed vole groups over the first 2-3 weeks, followed by a gradual drop in metabolic rate during the last weeks of exposure. We hypothesized that initial exposure would cause the HPT axis to compensate for any reductions in thyroid hormone secretion from the thyroid gland. An increase in circulating thyroid hormones

usually results in increased metabolic rate. With severe and/or prolonged perchlorate exposure and/or iodine deficiency in thyroid, HPT activation will be unable to maintain euthyroid status, causing decreased levels of circulating thyroid hormones and eventually reduced metabolic rate. This trend was not evident in our data over the six-week exposure period (Figure 1).

Since there have been no reports of any species of *Microtus* showing torpor either on a seasonal basis or for a shorter term, they must always expend energy for thermoregulation. Therefore, thermoregulation is a major maintenance cost for voles, as well as other rodent species with similar behavior (Tamarin, 1985). Perchlorate exposure had no effect on resting metabolic rate in this study, but that did not necessarily mean that it would have no effect on metabolic rate under conditions of cold exposure. The data do not show a marked reduction in the ability of exposed animals to deal with cold-stress tests. Again, it may require a higher dose or longer duration of exposure to see such an effect on cold-tolerance. Some of the voles in the high exposure group showed signs that they were able to deal with cold temperatures as well as control animals. Three of the seven voles in the high exposure group had metabolic rates higher than those of the mean control vole metabolic rate.

Reductions in thyroid hormones are often related to perchlorate exposure and thyroid hormones are key players in metabolic capacity, however, we found no relationship between peak metabolic rate and thyroxine concentrations and no significant differences in T_4 levels among groups; therefore, it may be possible that these animals were able to overcome any potential effects of exposure. Iodine was not limited in the rodent diet, as the rodent chow contains an average value of 1.1 ± 0.17 ppm iodine (Purina Mills International website). With sufficient iodine or iodide in the diet, the HPT axis should be able to maintain a euthyroid status (McNabb, 1992). Prairie voles also show a higher weight-specific rate of oxygen consumption in winter than in summer which may allow tolerance to lower thermal exposures (Wunder et al., 1977).

Our data did not show a marked reduction in plasma triiodothyronine. Our results are somewhat consistent for lower levels of perchlorate exposure (1.0 mg/kg), however our results contradict other studies that used a similar high exposure (10.0 mg/kg) for a different species (Siglin et al., 2000). Although the results were not statistically significant, we did find a dose-response pattern in plasma T_4 levels. Despite a reduction in T_4, T_3 is the biologically thyroid hormone and any reduction in T_4 may not have an impact on T_3 concentrations. A change in T_4 concentration that does not have an impact on deiodination processes would not impact the levels of T_3 (Hulbert et al., 1985). Cellular metabolism and the generation of ATP are tied to concentrations of T_3, which were not effected by perchlorate exposure. It should seem logical that resting metabolic rates as well

as peak metabolic rates would not be affected either. More exposure/dosing studies are needed with other rodent species, multiple treatments, and longer exposure times to determine if perchlorate can alter metabolic rates in endotherms.

Overall, this study did not provide evidence for energetic costs associated with perchlorate exposure at the individual level. Several issues must be taken into consideration before drawing any conclusions on whether or not perchlorate exposure can induce alterations in metabolic rate, and more importantly thermoregulatory capabilities. Before we can fully understand any mechanisms of perchlorate-induced alterations in metabolic rate in homeotherms, we must first understand how the HPT axis responds to perchlorate exposure at varying levels. One of the most popular indices of exposure has been plasma thyroid hormone level, which are probably the most variable measure of thyroid function. This is most likely due to the dynamic adjustment ability of the HPT axis (McNabb, 1992). Current research is proving that there are better techniques, such as thyroidal T content, for assessing perchlorate exposure, at least in avian species (McNabb et al., in press). Because animals often have the ability to adapt behaviorally or physiologically under conditions of toxicant stress, there should be an emphasis on the need for further evaluation of the interaction between perchlorate exposure and adaptive strategies. For instance, small mammals in temperate climates often compensate for thermal stress in winter by increasing their resting metabolic rate and activity of nonshivering thermogenesis (Tomasi et al., 1994). Rodents also respond to cold exposures by inducing growth of brown adipose tissue (BAT), the tissue that is responsible for the generation of nonshivering thermogenesis (Heldmaier, 1975). Given that rodents have these physiologically controlled adaptive responses to cold-conditions, it is possible that low, environmentally-relevant concentrations of perchlorate exposure will have little or no effect on an animal's ability to deal with cold-stress. However, avian species do not have BAT as an energy reserve for the production of heat in times of cold-stress. Birds that are most at risk, such as waterfowl and ground-dwelling birds, experiencing marked decreases in thyroidal hormone stores may be more at risk under cold-conditions than mammals. Given that endothermic organisms increase the rate of thyroid hormone secretion and sometimes the rate of thyroid hormone utilization under cold-stress (McNabb, 1994), severe depletion of thyroidal hormone stores would indicate that these animals would have little capability to respond to the condition.

16.0 References:

Banta, M.R. and D.W. Holcombe. 2002. The effects of thyroxine on metabolism and water balance in a desert-dwelling rodent, Merriam's kangaroo rat (*Dipodomys Merriami*). Journal of Comparative Physiology Part A. 185: 463-470.

Burger, M.F. and Denver, R.J. (2002). Plasma thyroid hormone concentrations in a wintering passerine bird: Their relationship to geographic variation, environmental factors, metabolic rate, and body fat. Physiological and Biochemical Zoology. 75(2): 187-199.

Danforth, E. and Burger, A. (1984). The role of thyroid hormones in the control of energy expenditure. Clinics in Endocrinology and Metabolism. 13(3): 581-595.

French JB, Volturea MB, Tomas TE. 2001. Effects of pre-and postnatal polychlorinated biphenyl exposure on metabolic rate and thyroid hormones of white-footed mice. Environmental Toxicology and Chemistry Vol 20(8): 1704-1708.

Haim, A. and Izhaki, I. (1993). The ecological significance of resting metabolic rate and non-shivering thermogenesis for rodents. J. Therm. Biol. 18(2): 71-81.

Heldmaier, G. (1975). The effect of short daily cold exposures on development of brown adipose tissue in mice. J. Comp. Physiol. 98: 161-168.

Hudson, J.W. and Kimzey, S.L. (1966). Temperature regulation and metabolic rhythms in Population of the house sparrow, *Passer domesticus*. Comp. Biochem. Physiol. 17: 203-217.

Hulbert, A.J., D.S. Hinds, and MacMillen, R.E. (1985). Minimal metabolism, summit metabolism and plasma thyroxine in rodents from different environments. Comp. Biochem. Physiol. 81, 687-693.

Marsh, R.L. and Dawson, W.R. (1989). Avian adjustments to cold. Advances in Comparative and Environmental Physiology. 4: 206-253.

McNabb, F.M.A. (1992). Thyroid hormones. Prentice Hall, Englewood Cliffs, NJ, USA.

McNabb, F.M.A. (1994). Thyroids. In *Sturkie's Avian Physiology*. 5th ed.

McNabb, F.M.A. (1995). Thyroid hormones, their activation, degradation, and effects on metabolism. Journal of Nutrition. 125: 1773-1776. Invited Symposium paper presented as part of the 59th Annual Poultry Nutrition Conference: Metabolic Modifiers, 78th FASEB meeting, April, 1994

McNabb, F.M.A, Larsen,C.T., and Pooler, P.S. (2004). Ammonium perchlorate effects on thyroid function and growth in bobwhite quail chicks. Envir Toxicol & Chem (in press).

Rosenmann M. and Morrison P. (1974). Maximum oxygen consumption and heat loss facilitation in small homeotherms by He-O_2. Am. J. Physiol. 226, 490-495.

Siglin JC, Matie DR, Dodd DE, Hildebrandt PK, Baker WH. 2000. A 90-day drinking water study in rats of the environmental contaminant ammonium Perchlorate. Toxicological Sciences. 57: 61-74.

Smith, P.N., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J. 2001. Preliminary Assessment of Perchlorate in Ecological Receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, TX. Ecotoxicology, 10: 305-313.

Tamarin, RH. 1985. Biology of New World *Microtus*. Special Publication No. 8. The American Society of Mammalogists.

Thuett KA, et al. 2002. In utero and lactational exposure to ammonium perchlorate in drinking water: effects on developing deer mice at postnatal day 21. Journal of Toxicology and Environmental Health. Part A. 65: 1061-1076.

Tomasi, T.E. and Mitchell, D.A. (1994). Seasonal shifts in thyroid function in the cotton rat, (*Sigmodon hispidus*). Jounal of Mammalogy, 75(2), 520-528.

United States Environmental Protection Agency. (1993). Wildlife Exposure Factors Handbook. Vol I. pp. 2-311-2-322.

Wunder BA, Dobkin DS, Gettinger RD. 1977. Shifts of thermogenesis in the prairie vole (*Microtus ochrogaster*), strategies for survival in a seasonal environment Oecologia 29: 11-26.

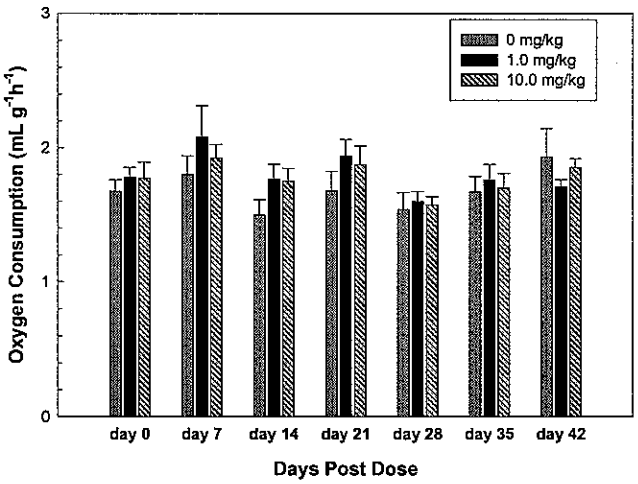


Figure 1. Weekly mean resting metabolic rates (ml g^{-1} h^{-1}) of prairie voles exposed to perchlorate in the drinking water (n=21).

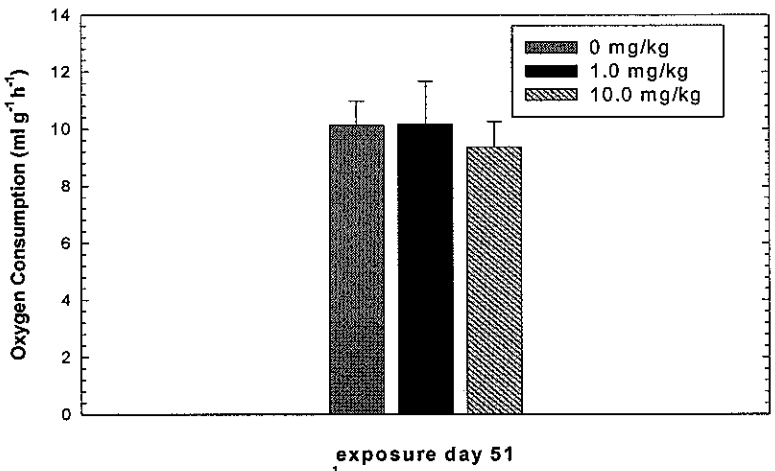


Figure 2. Peak metabolic rates (ml g^{-1} h^{-}1) of prairie voles exposed to perchlorate for 51 days. Temperature in metabolic chamber held at 12°C for 20 minutes followed by a drop in temperature to 5°C for a maximum duration of 60 minutes.

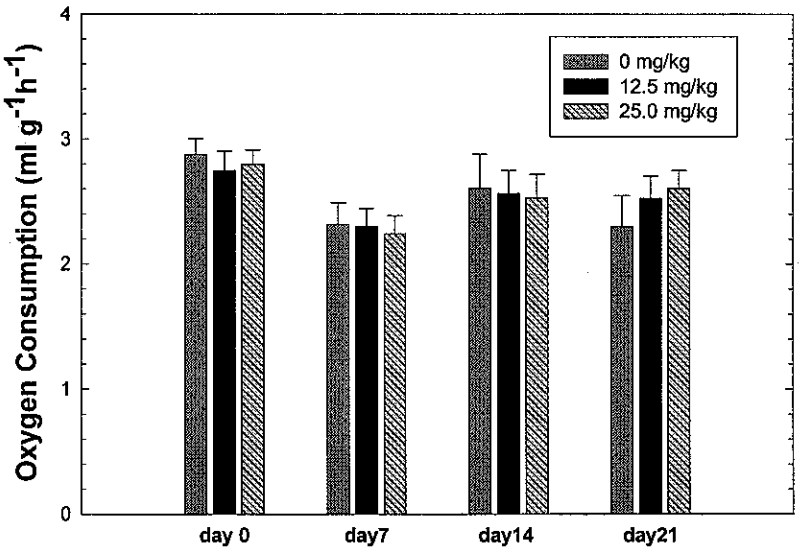


Figure 3. Weekly mean resting metabolic rates of house sparrows dosed with perchlorate through 21 days of dosing. Experiment terminated after 21 days of dosing due to the high occurrence of disease in the sparrow colony, thus compromising the results of the study.

Table 1. Mean prairie vole plasma T4 (ug/dL), T3 (ng/dL), and tissue weights (g/g b.w.) following 51 days of exposure to perchlorate in the drinking water.

Treatment	Plasma T4	PlasmaT3*	Liver (w.w.)	Kidney (w.w.)
0 mg/kg	3.01(0.83)	109.29(30.10)	0.045(0.006)	0.010(0.007)
1.0mg/kg	2.74(1.36)	94.83(32.71)	0.042(0.009)	0.010(0.007)
10.0 mg/kg	1.70(0.91)	95.57(13.23)	0.046(0.004)	0.011(0.001)

Numbers in parentheses are S.D.
*n=16

Table 2. Individual and mean house sparrow thyroxine concentrations (ug/dL) and liver and kidney masses (g) following 3 weeks of perchlorate dosing.

Treatment	ID	Plasma T4 [a]	Liver (w.w.)	Kidney (w.w.)
control	1c	0.2224	0.60	0.19
	2c	0.1647	0.79	0.18
	3c*	0.4229	0.67	0.15
	4c*	0.3988	1.38	0.25
	5c	0.3465	0.59	0.18
	6c [b]*	NA	NA	NA
	7c*	NA	1.06	0.18
			0.85(0.31)	0.19(0.03)
12.5mg/kg	1ld	NA	0.69	0.19
	2ld	NA	0.44	0.15
	3ld	ND	0.60	0.16
	4ld	0.0736	0.79	0.2
	5ld*	NA**	0.82	0.18
	6ld*	NA	0.80	0.24
	7ld*	NA	1.18	0.25
			0.76(0.23)	0.20(0.04)
25.0mg/kg	1hd*	NA	0.56	0.15
	2hd	ND	0.60	0.18
	3hd	ND	0.54	0.15
	4hd	ND	0.57	0.2
	5hd*	0.1061	0.57	0.16
	6hd*	NA	0.66	0.17
	7hd [b]*	NA	NA	NA
			0.58(0.04)	0.17(0.02)

Numbers in parentheses are S.D.
* diseased bird (House Finch Disease)
** death due to injury
[a] missing values for plasma T4 are due to lack of plasma or death before blood sampling
[b] tissues and plasma not collected
NA= not available
ND= not detectable

Table 3. Mean concentrations (ug/g d.w.) of perchlorate in house sparrow livers and kidneys following 3 weeks of perchlorate dosing.

Treatment	Liver ClO4	Kidney ClO4
0 mg/kg	0	0
12.5 mg/kg	57.41(68.77)	34.85(42.83)
25.0 mg/kg	234.48(258.26)	362.98(337.18)

Numbers in parentheses are S.D.

Project No.T9700

A STUDY PROTOCOL

ENTITLED

Perchlorate-Induced Alterations in Metabolic Rate and Thermoregulation in Small Mammals and Birds

STUDY/PROTOCOL NUMBER: MRT-03-01

SPONSOR:

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY:

Name/Address:

The Institute of Environmental & Human Health
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management:

Dr. Ronald J. Kendall
Director, TIEHH

Study Director:

Philip N. Smith

PROPOSED EXPERIMENTAL
START DATE: APRIL 1, 2003

1. DESCRIPTIVE STUDY TITLE:

Perchlorate-Induced Alterations in Metabolic Rate and Thermoregulation in prairie voles (*Microtus ochragaster)* and house sparrows (*Passer domesticus*)

2. STUDY NUMBER: MRT-03-01

3. SPONSOR:

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

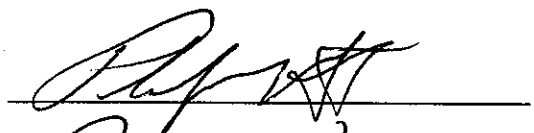
4. TESTING FACILITY NAME & ADDRESS:

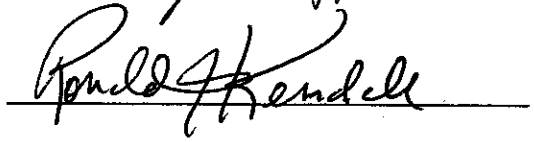
The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: April 1, 2003
Termination Date: December 31, 2003

6. KEY PERSONNEL:
Dr. Philip N. Smith, Study Director / Study Advisor
Mr. John Isanhart, co-investigator
Miss Jaclyn Canas, co-investigator
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Testing Facility Management

7. DATED SIGNATURES:


5/16/03 Dr. Philip N. Smith


5/17/03 Dr. Ron Kendall
Testing Facility Management

5-16-03

Mr. Ryan Bounds
Quality Assurance Manager

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Lubbock, TX 79409-1163

9. STUDY OBJECTIVES / PURPOSE:

- To evaluate the effects of perchlorate on metabolic rate and thyroid function in prairie voles and house sparrows through analysis of thyroid hormones, tissue perchlorate concentrations, and comparison of VO_2 between treatment and control groups; previous studies have shown elevated metabolic rates in thyroid hormone-administered birds and rodents (Banta and Homlcombe, 2002; Burger and Denver, 2002); depressed metabolic rates and circulating thyroid hormone concentrations coincide with hypothyroidism, which are often effects of perchlorate exposure
- To evaluate thermoregulatory costs associated with perchlorate exposure in prairie voles and house sparrows by measuring metabolic rate during cold stress experiments; thyroid hormones are the key controllers of metabolism that are necessary for maintenance of constant body temperature in homeothermic animals; any disruption of shivering or non-shivering thermogenesis in birds or rodents, respectively, may place them at a disadvantage when faced with highly variable temperature extremes

10. TEST MATERIALS:

Test Chemical name: ammonium perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure
Source: Sigma Aldrich

Reference Chemical name (positive control): L-thyroxine
CAS number: 51-48-9
Characterization: 1.0 and 2.0 mg thyroxine-releasing pellets for birds; 0.5 and 1.5 mg thyroxine-releasing pellets for voles
Source: Innovative Research of America

11. JUSTIFICATION OF TEST SYSTEM

The prairie vole is a ground-burrowing rodent found in the north and central plains of the United States and in southern Canada. Voles are largely herbivorous, consuming primarily green succulent vegetation, but also roots, bark, and seeds as well. These small, non-hibernating mammals living in the north temperate and boreal regions are faced with seasonal changes in their thermal environment. They must be able to adapt to both hot and cold extremes, otherwise, they may be at a disadvantage when stressed with a contrasting season or extreme daily temperature fluctuation. Animals need enough insulative and/or thermogenic capability to withstand low temperature exposure without becoming hypothermic, and they must cope with cold stress by behavioral avoidance and/or increased thermogenic capacity (Wunder et al. 1977). Small mammals have a limited ability in increasing their insulation, therefore behavioral avoidance and thermogenesis become the most important means for maintaining a relatively narrow range of body temperature. Deficiencies in thyroid hormones have been associated with a loss of mental alertness, slow heart rate, and increased sensitivity to cold. Therefore, perchlorate-induced alterations in thyroid hormones may have secondary effects on metabolic rate and thermoregulation.

The house sparrow is a common passerine species that is often used as an avian behavioral and toxicological model. Birds, especially passerine species, are often used as sentinel species when assessing the ecological impacts of chemicals on ecosystems and populations. The insulative needs of birds are often met by a combination of plumage characteristics, behavioral avoidance of cold exposure, and shivering thermogenesis (increased metabolic rate). Subcutaneous fat tends to be more localized in birds than in mammals, therefore birds cannot rely on fat storage for insulation. Incubation in females, as well as males, can also potentially expose the incubating parent to extremely demanding thermal conditions. Altered thyroid hormone concentrations influence the overall metabolic energy supply, disrupt liver glycogen storage, and decrease plasma glucose levels. Cold temperatures tend to increase thyroid hormone secretion rates, and decreases in iodide transport into the thyroid gland from the blood tend to decrease thyroid hormone secretion rates (McNabb, 1995). Therefore, an association may exist between perchlorate reductions in thyroid hormone, as well as the blocking of iodide uptake into the thyroid gland by perchlorate, and decreased metabolic rates in animals.

Available toxicity data suggests that rodents may be sensitive to low levels of perchlorate in the environment (Thuett et al. 2002; Siglin et al. 2000). The United States Environmental Protection Agency also noted a need for the determination of the effects of dietary exposure to perchlorate in birds. Past research has shown that perchlorate administration at similar concentrations increased thyroid weights, decreased thyroid hormone levels, and increased thyroid follicular hyperplasia (Siglin et al. 2000). Dr. Anne McNabb of Virginia Tech has noted perchlorate-induced alterations in circulating thyroid hormones of adult and young northern Bobwhite quail (studies ongoing). Not only has perchlorate been detected by Texas Tech University researchers at high concentrations in water, but it has also been reported in plant vascular tissues and seeds at concentrations ranging from approximately 1 ppm to as high as 5000 ppm (Smith et al. 2001; data on file, TIEHH). Small mammal species and passerine birds may be found in such contaminated environments; therefore such species represent potentially useful models for assessing the ecological impacts of perchlorate exposure.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system)**:**

Species: Prairie voles (*Microtus ochragaster)* and House sparrow (*Passer domesticus*)

Strain:

Age: voles (21 - >100 days); birds (hatching year or after hatching year)

Number: approximately: voles (51); birds (50)

Source: voles (Texas Tech University vole breeding colony); birds (captured from Lubbock, TX)

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All voles will be placed in standard rodent cages and each cage will be labeled with a note card containing the appropriate identification information for the animal. Birds will be placed into individual labeled cages containing the appropriate identification information for the animal on the front of the cage. All birds will also be color banded, with a unique color code being assigned for each bird. Collected samples will be placed in individually labeled bags/containers and stored appropriately according to TIEHH SOP IN-3-02.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Oxygen consumption and carbon dioxide respiration will be determined for voles and sparrows that have been dosed with perchlorate in the drinking water and in perchlorate-injected worms, respectively, as well as for control groups. A positive control experiment will involve implanting thyroid hormone pellets into animals and comparing metabolic rates between groups. Metabolic rates will be compared between perchlorate dosing groups to detect for physiological responses. Concentrations of thyroid gland hormones and blood and tissue perchlorate will also be examined to evaluate for thyroid hormone imbalance and dose-related responses. Abilities of animals to deal with cold-exposure will be related to perchlorate exposure and thyroid hormone concentrations. Animals will be randomly assigned into dosing groups, as well as randomly assigned to a chamber for metabolic rate measurements. Voles and birds will be will assigned to an unlabeled cage and a random number table will be read, with every third number being considered. The number 1 will designate a control animal, a number 2 will designate an animal in the low dose group, and a number 3 will designate an animal in the high dose group. When a dosing group becomes filled, that number designating that group will no longer be considered and only the two remaining numbers will count. Numbers will be read until all animals are assigned to dose groups.

15. METHODS:

Metabolic Rate

The procedure for collecting oxygen consumption rates (acclimation period, positive control and perchlorate experiment) is as follows: One animal from each group (control, low dose, and high dose) will be placed in a respirometry chamber. Tests will be conducted between 0800 and 2200 hours. A 4-channel respirometry instrument (Qubit Systems, Ontario, Canada) with an open-flow configuration will be used. Individuals will be weighed at the beginning and end of testing. Animals will be disturbed as little as possible, as our whole purpose is to have them as calm as possible at the time of testing. Animals will then be placed in a cylindrical respirometry chamber. These chambers provide the animal with space to position itself without being cramped, while restricting it so it is not able to hurt itself. The chamber is made of borosilicate glass with each end consisting of a plastic end cap, one with a built-in electrical fan, the other with a small hole for the insertion of a temperature probe. Chambers will be placed in a temperature-controlled cabinet and animals will be allowed an initial 30 minutes to one-hour acclimitization period in the chamber to allow them to achieve a resting state. Air will be passing through each chamber at a rate of approximately 250-1000 ml/min during this period, as well as the test period. Testing will commence by collecting carbon dioxide and oxygen measurements at 20 minute intervals per channel, with a reference channel measurement between each sample measurement. Total run time (high estimate)= 60

minutes acclimitization + (20 minutes per channel x 6 samples per round)=180 minutes. Behavioral criteria will be used to determine whether animals are inactive during measurment of oxygen consumption to insure consistency of animals. Activity levels will also be monitored on the computer screen, with an increase in activity resulting in increased oxygen consumption. At the conclusion of each run, all animals will be placed back into assigned cages. This type of format will continue for 3-8 weeks (depending on which experiment), once a week, for all animals. Some sessions will be video-taped.

Summit Metabolic Rate (cold-stress)

Cold stress tests will be performed at the conclusion of resting metabolic testing. Tests will be performed on the same individuals that were used in the resting metabolic testing part of the perchlorate experiment. Lighting conditions will be kept constant throughout the study, and the major procedures will be conducted as stated previously, with the exception of a few minor adjustments. Tests will be performed between 0800 and 2200 hours. Initial core body temperatures will be taken by inserting a copper-constantan thermocouple into the colon/cloaca of the animal. Summit metabolic rate will be measured using helox gas (80% He, 20% oxygen) at a temperature of 5-15°C, or by placing animals in an environmental chamber that has been cooled to a temperature near freezing. One animal from each group (control, low dose, or high dose) will be tested at a time, instead of three at a time for this procedure. Each animal will be tested for a maximum of 60 minutes at a time. Those animals that become hypothermic relatively quickly and have uncharacteristically low metabolic rates will be removed from the chamber and monitored for signs of distress. Hypothermia is determined by a steady state decline in the volume of oxygen consumed over several minutes. Upon removal from the chamber, colonic/cloacal temperature will be measured with a thermocouple and animals will be placed back into assigned cages.

15.1 Test System acquisition, quarantine, acclimation

Voles will be acquired from the vole colony at the Human Sciences Laboratory Animal Facilities at TTU. Voles will be provided with adequate care and be given a 1-2 week acclimation period to determine how they will adjust to their new environment and procedures of taking oxygen consumption measurements.

Birds will be captured from various locations in the Lubbock, TX area, transferred to animal facilities, and individually housed in cages. Sparrows will be provided with adequate food, water, etc. and treated for parasites. All animals will be allowed 1-2 weeks to acclimate to their new environment, and daily observations will be made for any unusual behavior or illness.

15.2 Test Material Application

Concentrations, Frequency, Method of Application:

Thyroxine:
A L-thyroxine hormone releasing pellet (1.5 mm diameter) will be subcutaneously implanted into the lateral side of the neck (between ear and shoulder, or ear and base of neck) in each animal involved in the positive control dosing experiment for both birds and rodents. Before implantation, animals will be sedated with a Halothane soaked cotton ball in a desiccating chamber. After determining that the animal is sedated, it will be removed from the chamber and the pellet implanted before the animal awakens. Pellets are implanted with a 10 gauge trochar, which is a stainless steel precision tool with a regular medical point needle and rounded stylet protruding 1/8" from needle point for easy implantation of small pellets up to 3mm in diameter. Trochars will be appropriately sterilized by dipping them into a 2% chlorhexidine solution between animals and then rinsing the trochar with milli-Q water to remove any of the disinfectant residue. The pellets release hormone continuously over a 21 day period. Voles in the low dose group will receive a 0.5 mg pellet (daily dose = 0.0238 mg/day), and the high dose group will receive a 1.5 mg pellet (daily dose = 0.0714 mg/day). Control groups will receive a placebo pellet. Birds in the low dose group will receive a 1.0 mg pellet, the high dose group will receive a 2.0 mg pellet, and the control group a placebo pellet.

Perchlorate:
Voles will be given perchlorate dissolved in drinking water at doses of 1.0 mg/kg (low dose group) and 10 mg/kg (high dose group). Birds will be given two waxworms or mealworms injected with perchlorate at a daily dose of 12.5 (low dose) and 25.0 mg/kg bird body weight (high dose). Birds will receive one worm in the morning and one in the afternoon. Control birds will receive worms injected with milli-Q water. Dosing will be carried out for a total of 6-8 weeks for both voles and birds.

Justification for Exposure Route:

The advantages of thyroxine releasing pellets include reduction in animal handling and trauma, enhancement of experimental efficiency, and insurance of proper dosing. The problem with putting thyroxine in water or food is contamination of food and cage surfaces leading to difficult clean-up. Injection or intubation would be too stressful on such small animals.

For voles, perchlorate will be dissolved in the drinking water as this allows for the least stressful and easiest type of administration. There is of course individual variation in the

perchlorate dose this way, but along with exposure from contaminated food, it is probably the approach closest to what happens in the wild.

Birds will be exposed by being fed perchlorate-injected worms. This route provides the most precise and accurate method for exposing birds to perchlorate. This method has the advantage of no handling effects and no spreading of perchlorate around the cage area.

Exposure Verification:

Each dosing solution will be verified for ammonium perchlorate concentration by ion chromatography (TIEHH SOP GW-02-03).

15.5 Animal Sacrifice and Sample Collections

Animals will be weighed and anesthetized in a saturated carbon dioxide chamber. Blood samples will then be collected from each vole (TIEHH SOP ET 03-19) and bird (TIEHH SOP IN 3-08-01), placed into heparinized microcentrifuge tubes, and centrifuged until plasma has been separated. Plasma will then be transferred to labeled tubes and frozen until analysis. All animals will then be euthanized (TIEHH SOP AF-01-03) and necropsied (TIEHH SOP IN-03-01). All animals will be monitored until respiration and cardiac function has ceased. Tissue samples collected (e.g. liver, kidney, etc.) will be frozen and may be used for residue analysis. Thyroids will be collected, digested, and processed for the collection of thyroid hormones.

15.6 Endpoint Analysis

The main study endpoint is to determine if perchlorate-related reductions in thyroid hormones elicit changes in metabolic rate in animals. Study endpoints consistent with behavioral effects include general ability of animals to deal with cold exposure, adult composure, and time of conversion from normal behavior to any noticeable change. Biochemical endpoints may include circulating thyroid hormones in animal plasma (TIEHH SOP MT-02-02), thyroidal hormone content, and concentrations of perchlorate in specific tissues (TIEHH SOP AC-02-15). SOPs for measurement of metabolic rate (RMR and summit), thyroidal hormone content, and measurement of internal body temperature will be developed after these procedures have been optimized. Other endpoints include measurement of oxygen consumption rate, carbon dioxide production, and internal body temperature after cold exposure.

16. PROPOSED STATISTICAL METHODS

Effects of dosage on body mass, tissue mass, tissue-perchlorate concentrations, and hormone concentrations will be tested with analysis of variance. Fisher's protected least-square difference procedure will be used *a posteriori* to look at the differences between group means. The effect of dosage on oxygen consumption rates and temperature regulation over time will be tested with repeated measures analysis of variance.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include daily food and water consumption data, morphological measurements, sample collection logs, analytical data, and hormone analysis data.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- thyroid hormone concentrations
- metabolic rate measurements
- plasma and tissue perchlorate concentrations
- general abilities of animals to deal with cold exposure
- internal body temperatures after cold exposure

Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Department of Defense/Strategic Environmental Research and Development Program. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Banta, M.R. and D.W. Holcombe. 2002. The effects of thyroxine on metabolism and water balance in a desert-dwelling rodent, Merriam's kangaroo rat (*Dipodomys Merriami*). Journal of Comparative Physiology Part A. 185: 463-470.

Burger, M.F. and Robert J. Denver. 2002. Plasma Thyroid Hormone Concentrations in a Wintering Passerine Bird: Their relationship to Geographic Variation, Environmental Factors, Metabolic Rates, and Body Fat. Physiological and Biochemical Zoology. 75(2): 187-199.

McNabb, F.M.A. 2000. Thyroids. In Sturkie's Avian Physiology, 5th ed. Editor G. Causey Whittow. Pg. 461-471.

Siglin JC, Matie DR, Dodd DE, Hildebrandt PK, Baker WH. 2000. A 90-day drinking water study in rats of the environmental contaminant ammonium Perchlorate. Toxicological Sciences. 57: 61-74

Smith, P.N., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J. 2001. Preliminary Assessment of Perchlorate in Ecological Receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, TX. Ecotoxicology, 10: 305-313.

U.S. EPA. 2002. Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization.

Wunder BA, Dobkin DS, Gettinger RD. 1977. Shifts of thermogenesis in the prairie vole (Microtus ochrogaster), strategies for survival in a seasonal environment Oecologia 29: 11-26.

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __x__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __x__ Protocol ____ SOP ____ Other _____
Title: Perchlorate-Induced Alterations in Metabolic Rate and Thermoregulation in Small Mammals and Birds
Dated: 1/27/04
Document # (if appropriate): MRT-03-01
Page #(s): 6-10
Section #: 15.0
Text to reference:
1. "Tests will be conducted between 0800 and 2200 hours."
2. "Animals will then be placed in a cylindrical respirometry chamber." "The chamber is made of borosilicate glass with each end consisting of a plastic end cap, one with a built-in electric fan, the other with a small hole for the insertion of a temperature probe."
3. "Air will be passing through each chamber at a rate of approximately 250-1000 ml/min during this period as well as the test period."
4. "Testing will commence by collecting carbon dioxide and oxygen measurements at 20 minute intervals per channel, with a reference channel measurement between each sample measurement."
5. "Total run time (high estimate) = 60 minutes acclimatization + (20 minutes per channel x 6 samples per round) = 180 minutes."
6. "Tests with be performed between 0800 and 2200 hours."
7. "Lighting conditions will be kept constant throughout the study, and the major procedures will be conducted as stated previously, with the exception of a few minor adjustments."

Change in Document:
1. "Tests will be conducted between 0730 and 1700 hours for voles and 2030 and 0630 for sparrows."
2. "Voles will be placed into a modified plastic rodent cage with a volume of approximately 2.3 liters, and birds will be placed into 2.89 liter paint cans that have been modified into respirometry chambers."
3. "Air will be passing through each chamber at a rate of approximately 850-1000 ml/min during this period, as well as the test period."

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

4. "Testing will commence by collecting carbon dioxide and oxygen measurements at 15 minute intervals per channel, with a reference channel measurement between each sample measurement."
5. "For voles, total run time (high estimate) = 60 minutes acclimatization + (15 minutes per channel x 6 samples per round x 5 rounds) = 510 minutes. For birds, total run time will be a maximum of 10 hours."
6. "Tests will be performed at times previously stated."
7. "Voles will be placed into a borosilicate glass chamber with each end consisting of a plastic end cap, one with a built-in electrical fan, the other with a small hole for the insertion of a temperature probe. Birds will be placed in the same chambers used for resting metabolic rate experiments. Each animal will be tested for a maximum of 60 minutes at a time."

Section #: 15.2
Text to reference:
8. "A L-thyroxine hormone releasing pellet (1.5 mm diameter) will be subcutaneously implanted into the lateral side of the neck (between ear and shoulder, or ear and base of neck) in each animal involved in the positive control dosing experiment for both birds and rodents."
9. "Birds in the low dose group will receive a 1.0 mg pellet; the high dose group will receive a 2.0 mg pellet, and the control group a placebo pellet."
10. "Birds will be given two waxworms or mealworms injected with perchlorate at a daily dose of 12.5 (low dose) and 25.0 mg/kg bird body weight (high dose). Birds will receive one worm in the morning and one in the afternoon. Control birds will receive worms injected with milli-Q water."

Change in Document:
8. "A L-thyroxine hormone releasing pellet (1.5 mm diameter) will be subcutaneously implanted into the lateral side of the neck (between ear and shoulder, or ear and base of neck) in each vole involved in the positive control dosing experiment."
9. Procedure no longer in use.
10. "Birds will be dosed with perchlorate in the drinking water at a daily dose of 12.5 (low dose) and 25.0 mg/kg bird body weight (high dose). A 200 uL (Gilson Pipetman) pipette will be used to dose birds with 100 uL of the appropriate dosing solution. The birds will be orally dosed by placing the solutions on the side of the bill and allowing for ingestion. Control voles and sparrows will receive milli-Q water."

* Sequentially numbered in order of the date that the change is effective

TIEHH
Box 41163
Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Change In Study Documentation Form

Section #: 15.3
Text to reference:
11. "Birds will be exposed by being fed perchlorate-injected worms. This route provides the most precise and accurate method for exposing birds to perchlorate. This method has the advantage of no handling effects and no spreading of perchlorate around the cage area."

Change in Document:
11. "Dosing birds by the previously stated method is the most precise way to orally dose a bird without over-stressing it. Each bird is handled for less than 30 seconds, and the proposed dosing is less stressful than gavage."

Section #: 17
Text to reference:
12. "- thyroid hormone concentrations"
13. "- plasma and tissue perchlorate concentrations"

Change in Document:
12. "- thyroid hormone concentrations (plasma and thyroid)"
13. "- tissue perchlorate concentrations"

Justification and Impact on Study:
The proposed changes in the study are necessary as several methods needed improvement or added steps, as the original methods stated in the first draft of the study protocol would not suffice. Data collection procedures are now more efficient and provide more precise data, and the avian dosing methods have been improved because original methods did not work. Birds were not given thyroxine-releasing pellets because implantation procedures were too dangerous on such a small bird. Other methods of dissolving the thyroxine free acid proved to be ineffective as the solvent affected the bird metabolic rate as well.

Submitted by: Signature: _____ Date: 01/30/04

Authorized by: Study Director: _____ Date: 1/30/04

Received by: Quality Assurance Unit: _____ Date: 1/30/04

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: _____
*Change No: _____
Page: _____ of _____

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: __x__ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __x__ Protocol ____ SOP ____ Other _____
Title: Perchlorate -Induced Alterations in Metabolic Rate and Thermoregulation in Small Mammals and Birds
Dated: 08 October 2003
Document # (if appropriate): MRT –3-01
Page #(s): page 8 of 11
Section #: 15.2
Text to reference: Birds will be given two waxworms or mealworms injected with perchlorate at a daily dose of 12.5 (low dose) and 25.0 mg/kg bird body weight (high dose). Birds will receive one worm in the morning and one in the afternoon. Control birds will receive worms injected with milli-Q water.

Change in Document: Birds will be given an oral dose of ammonium perchlorate water_at 12.5 and 25.0 mg/kg bird body weight. Liquid will be administered with a pipette and placed on the edge of the lower mandible, thus allowing the bird to drink the dosing solution. Birds will be dosed twice daily, once in the morning and once in the afternoon. Control birds will receive milli-Q water.

Justification and Impact on Study: After initial experiments, we discovered that birds may stop eating mealworms after several days of dosing. This presents problems with the dosing regimen; therefore the best alternative is to dose the birds using the pipette method described above.

Submitted by: Signature: _____ Date: 10/08/03

Authorized by: Study Director: _____ Date: 10/9/03

Received by: Quality Assurance Unit: _____ Date: 10/8/03

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

Uptake of Perchlorate in Invertebrates

STUDY NUMBER: INV-03-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

RESEARCH INITIATION: 02/28/2003

RESEARCH COMPLETION: 12/31/2003

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GOOD LABORATORIES PRACTICES STATEMENT

Project AQUA 03-01, entitled " Perchlorate in Invertebrates, Periphyton, and Detritus at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/26/04
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

Ryan Bounds
Quality Assurance Manager

March 25, 2004
Date

1. **DESCRIPTIVE STUDY TITLE:** Uptake of Perchlorate in Invertebrates

2. **STUDY NUMBER:** INV-03-01

3. **SPONSOR:**

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. **TESTING FACILITY NAME & ADDRESS:**

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-41163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**

Start Date: (date of chemical application) June 25, 2003

Termination Date: (date of last data collected) December 31, 2003

6. **KEY PERSONNEL:**

Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7. **STUDY SUMMARY:**

Ramshorn snails (*Heliosoma spp.*) and blackworms (***Lumbriculus variegatus***) were exposed to 100 mg/L sodium perchlorate for 1-5 days. Perchlorate concentrations were then determined in both of these species after various periods of time. The snails reached steady state concentrations within 1 day, and the bioconcentration factor was 0.26. In contrast, blackworms reached steady state by 3 days, and the bioconcentration factor was 0.84. However, the steady state concentration for blackworms was not significantly different from 100 mg/L (the water concentration), so the bioconcentration factor may indeed be 1.0 for this species. These data indicate that fish that feed upon snails may not be exposed to as much perchlorate from food as do fish that feed on *L **variegatus***.

8. **STUDY OBJECTIVES / PURPOSE:**

To determine kinetics of uptake of perchlorate in invertebrate species.

9. TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: Aldrich Chemical Company

Reference Chemical name:
ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts were added.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in worms, snails, and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Comparative analysis of perchlorate accumulation in fish and water from field-collected specimens indicates that perchlorate levels may be higher in fish than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish. This suggests that worms and snails in the field are being exposed by some route other than direct absorption from the water. Possibly fish are being exposed through the food chain. In addition, many fish in stream habitats, especially the ones that have been analyzed so far, feed on aquatic invertebrates. Uptake and possible accumulation from the water has not yet been addressed in invertebrates.

11. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system)**:**

Species: Blackworms (***Lumbriculus Variegatus***), ramshorn snails (*Heliosoma spp.*)

Strain: Feral organisms or bred in hatcheries

Age: Adults.

Number: Approximately 100 grams, wet weight, of each species

Source: Purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Beakers or glass jars were labeled with the project number, test system, date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Worms and snails were exposed to 100 ppm sodium perchlorate in precleaned beakers. Beakers were cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Beakers for Perchlorate Assays". For exposures, beakers were located on shelves capable of supporting such weight. The experimental design consisted of a randomized block design, with each shelf constituting a block. The arrangement of the beakers within each block was randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the beakers within each block was determined by a random number generator, random number table or by rolling dice. Each block contained at least 1 container of each treatment. Worms and snails were placed in the beakers in random order, within blocks, using the procedure described below. Worms and snails were exposed in separate beakers.

14. METHODS:

14.1 Test System acquisition, quarantine, acclimation

Worms and snails were obtained from commercial vendors. Upon arrival to the lab, they were acclimated in clean aquarium water for 2 days. Clean water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Once acclimated, worms and snails were exposed to sodium perchlorate dissolved in water. One hundred percent water changes were carried out every other day by pouring the water out and adding fresh clean water.

14.2 Test Material Application

Exposures began after worms and snails were acclimatized. Worms and snails were placed into beakers or beakers containing 100 ppm sodium perchlorate, with approximately 5 g of worms or snails per aquarium and 5 replicate beakers per treatment. A stock solution of 100 g/L perchlorate in reconstituted fresh water was used to dose the worms and snails. The beakers were filled with 1.5 L water, and 1.5 ml of stock solution were added according to the desired concentration of the aquarium water. Every other day, debris 100% of the water were replaced in each tank with undosed water (as described in 15.1), and perchlorate stock solution were added to maintain the desired concentration.

Rates/concentrations: Worms and snails were exposed to 100 ppm perchlorate in water.

Frequency: Five replicate beakers of each concentration for each species were continually exposed for 1, 2, 3, 4 (snails only) and 5 days.

Route/Method of Application: Route was via dermal, oral and respiratory exposure as the chemical were in the beaker/beakers water.

Stock solutions for the study were mixed in precleaned glass beakers as indicated in SOP AQ-1-02. Stock solutions were made by dissolving sodium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts or equivalent, in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of sodium perchlorate compound were weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH were checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted, if necessary, as above.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because worms and snails respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

14.3 Test System Observation

Beakers were observed on a daily basis. The number of individuals that expire each day were recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager were determined at least 3 times per week.

.

14.4 Animal Sacrifice and Sample Collections

Invertebrates were killed by immersion in 100% ethanol until dead. Individuals used for perchlorate analysis were air-dried for at least 3 days and stored in plastic storage bags. Perchlorate concentration in tissues were extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Animals were pooled to obtain sufficient tissue for analysis (a minimum of approximately 5 g for perchlorate).
Labeling: samples were labeled with a unique ID number according to the following scheme:

Species (2 letter abbreviation) – trial number - exposure duration (days)- sample number.
Species abbreviations will by HS for ramshorn snails (*Heliosoma*) and LV for blackworms

E.g., LV-1 –2 – 3 blackworm sample #3, 2-day exposure, trial # 1.

14.5 Endpoint analysis

Perchlorate in tissues was extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in aquarium water or extracted from tissues was according to SOP AC-2-11 "Analysis of Perchlorate by IC".

15 STATISTICAL METHODS

All data were checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances wad checked using Bartlett's or Lavine's test. Comparisons between treatments was accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons.

16 RESULTS

The body burdens of the snails were not statistically different between days (Fig. 1). These data indicate that the snails reached steady state concentrations within 1 day, and the bioconcentration factor was 0.26. In contrast, blackworms reached steady state by 3 days, and the bioconcentration factor was 0.84. However, the steady state concentration for blackworms was not significantly different from 100 mg/L (the water concentration; $p > 0.05$, Student's t-test), so the bioconcentration factor may indeed be 1.0 for this species.

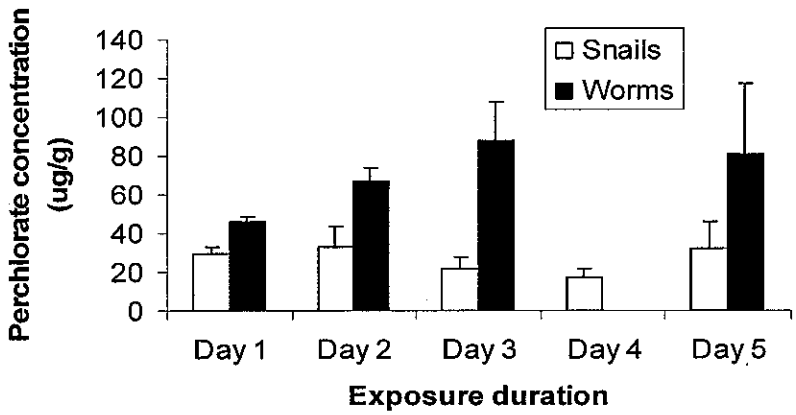


Figure 1 - Perchlorate concentrations in ramshorn snails and blackworms after 1-5 days of exposure.

17 DISCUSSION

The results reported herein indicate that perchlorate can be taken up by invertebrates from the water, but, contrary to field studies, perchlorate steady state concentrations were less than that

found in the water. Thus, these data provide no additional insight as to the findings that concentrations of perchlorate in fish or their invertebrate prey are often greater than concentrations found in the water in field situations (e.g., Smith et al. 2001). The alternative hypothesis to explain these findings is that 1) perchlorate water concentrations in contaminated streams are highly variable, and may consist of pulses of higher concentrations followed by periods of low or no perchlorate release into the water and 2) the elimination of perchlorate from biological tissues lags behind the rate of clearance of perchlorate from the water column, so that perchlorate may be found in biological tissues after it has decreased to non-detectable levels in the water.

These data also indicate that there may be species-specific differences as to the rate of uptake and steady state bioconcentration factors. In snails, steady-state is reached fairly rapidly and the steady-state concentrations in the tissues are about ¼ that in the water. In the worms, on the other hand, steady state is reached after 3 days, and the steady-state concentrations are about 4 times higher than in snails. This may result in greater food-chain exposures to fish that feed on benthic worms than those that feed on snails.

18 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

19 REFERENCES:

Smith, N.S., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J., 2001. Preliminary assessment of pechlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

20 APPENDICES:

Study Protocol
Changes to Study Documentation

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Uptake of Perchlorate in Invertebrates

STUDY NUMBER: INV-03-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: June 25, 2003

1. **DESCRIPTIVE STUDY TITLE:** Uptake of Perchlorate in Invertebrates

2. **STUDY NUMBER:** INV-03-01

3. **SPONSOR:**

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. **TESTING FACILITY NAME & ADDRESS:**

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-41163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**

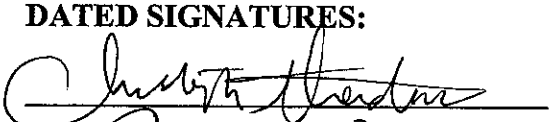
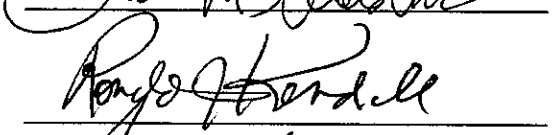
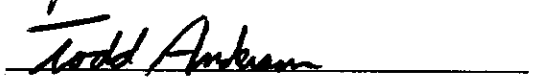
Start Date: (date of chemical application) June 25, 2003

Termination Date: (date of last data collected) December 31, 2003

6. **KEY PERSONNEL:**

Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7. **DATED SIGNATURES:**

	6/23/03	Dr. Christopher Theodorakis Study Director
	6/24/03	Dr. Ronald Kendall Testing Facility Management
	6-23-03	Mr. Ryan Bounds Quality Assurance Manager
	6-23-03	Dr. Todd Anderson Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT:

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. Ronald Kendall
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-211163

9. STUDY OBJECTIVES / PURPOSE:

To determine kinetics of uptake of perchlorate in invertebrate species.

10. TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 7601-89-0
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: Aldrich Chemical Company

Reference Chemical name:
ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts will be added.

11. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in worms, snails, and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Comparative analysis of perchlorate accumulation in fish and water from field-collected specimens indicates that perchlorate levels may be higher in fish than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish. This suggests that

worms and snails in the field are being exposed by some route other than direct absorption from the water. Possibly fish are being exposed through the food chain. In addition, many fish in stream habitats, especially the ones that have been analyzed so far, feed on aquatic invertebrates. Uptake and possible accumulation from the water has not yet been addressed in invertebrates.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system)**:**

Species: Blackworms (***Lumbriculus Variegatus***)**,** ramshorn snails (*Planorbis spp.*)

Strain: Feral organisms or bred in hatcheries

Age: Adults.

Number: Approximately 100 grams, wet weight, of each species

Source: Purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
The test system will consist of laboratory exposures constructed according to the experimental design described below. Beakers or glass jars will be labeled with the project number, test system, date of collection, concentration, and person responsible.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Worms and snails will be exposed to 100 ppm sodium perchlorate in precleaned beakers or mason jars ("containers"). Containers will be cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Containers for Perchlorate Assays". For exposures, containers will be located on shelves capable of supporting such weight. The experimental design will consist of a randomized block design, with each shelf constituting a block. The arrangement of the containers within each block will be randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the containers within each block by a random number generator, random number table or by rolling dice. Each block will contain at least 1 container of each treatment. Worms and snails will be placed in the containers in random order, within blocks, using the procedure described below. Worms and snails will be exposed in separate containers.

15. **METHODS:**

15.1 **Test System acquisition, quarantine, acclimation**

Worms and snails will be obtained from commercial vendors. Upon arrival to the lab, they will be acclimated in clean aquarium water for 2 days. Clean water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Once acclimated, worms and snails will be exposed to sodium perchlorate dissolved in water. One hundred percent water changes will be carried out every other day by pouring the water out and adding fresh clean water.

15.2 **Test Condition Establishment**

Exposures will begin after worms and snails have become acclimatized.

15.3 **Test Material Application**

Worms and snails will be placed into beakers or containers containing 100 ppm sodium perchlorate, with approximately 5 g of worms or snails per aquarium and 5 replicate containers per treatment. A stock solution of 100 g/L perchlorate in reconstituted fresh water will be used to dose the worms and snails. The containers will be filled with 1.5 L water, and 1.5 ml of stock solution will be added according to the desired concentration of the aquarium water. Every other day, debris 100% of the water will be replaced in each tank with undosed water (as described in 15.1), and perchlorate stock solution will be added to maintain the desired concentration.

Rates/concentrations: Worms and snails will be exposed to 100 ppm perchlorate in water.

Frequency: Five replicate tanks of each concentration will be continually exposed for 1, 2, 5, and 10 days.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/containers water.

Stock solutions for study will be mixed in precleaned glass containers as indicated in SOP AQ-1-02. Stock solutions will be made by dissolving sodium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts or equivalent, in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of sodium perchlorate compound will be weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH will be checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted, if necessary, as above.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because worms and snails respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water will be collected whenever animals are removed from the aquarium for analysis. The concentration of perchlorate in the water will be tested using ion chromatography.

15.4 **Test System Observation**

Containers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 **Animal Sacrifice and Sample Collections**

Invertebrates will be killed by immersion in 100% ethanol until dead. Individuals used for perchlorate analysis will be wrapped in aluminum foil or placed in cryogenic tubes suitable for liquid-phase liquid nitrogen and will be frozen by immersion in liquid nitrogen. Alternatively, dead animals may be air-dried for at least 3 days and stored in plastic storage bags. Perchlorate concentration in tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Animals may be pooled to obtain sufficient tissue for analysis (a minimum of approximately 5 g for perchlorate).

Labeling: samples will be labeled with a unique ID number according to the following scheme:

LP (laboratory perchlorate exposure) – species (2 letter abbreviation) - sample number. Species abbreviations will by SN for ramshorn snails and BW for blackworms

E.g., LP- SN- 0001- is the liver sample from snail sample # 0001.

If samples are to be divided into subsamples, then a suffix is attached. E.g., if the liver sample above is divided into 3 subsamples, these subsamples will be labeled:

LP-SN-0001.1
LP-SN-0001.2
LP-SN-0001.3

If samples are to be composited, then the prefix will be LPC. (e.g., LPC-BW-001 is worm composite # 1). The number of individual samples (snails only) and weight of individuals comprising the composite should be indicated on the dissection/tissue collection form and/or in a bound laboratory notebook.

Minimum information to be included on the label is project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information can include species, date collected and sex (if known), in decreasing order of importance. The number of days for which each sample was exposed to perchlorate will also be included on the form on in the laboratory notebook.

15.6 **Endpoint Analysis**

Perchlorate concentration in tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in aquarium water or extracted from tissues will be according to SOP AC-2-11 "Analysis of Perchlorate by IC".

16. PROPOSED STATISTICAL METHODS:

To statistically determine the differences between treatments in terms of histological endpoints, thyroid hormone or perchlorate body concentrations, 2-way ANOVA will be used to determine effects of concentration and time of exposure. Correlation and regression analysis may also be used to determine the relationship between response (body burden, thyroid function) vs. dose or vs. time.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Relative tissue distribution in bullhead catworms and snails, relationship between perchlorate body burden and exposure concentration, and preliminary data on thyroid function and/or histology will be included in the report.
Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses:

Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. **RECORDS TO BE MAINTAINED / LOCATION:**
The final report will be delivered to the Sponsor on or before March 20, 2004. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and a copy of the final report shall be archived at the testing facility.

19. **QUALITY ASSURANCE:**
The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. **PROTOCOL CHANGES / REVISIONS:**
All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

21. **REFERENCES:**
Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment _x___Deviation ____ Addendums

Document Reference Information
Check One: _x___ Protocol ____ SOP ____ Other _____
Title: ____ Perchlorate Uptake in Invertebrates
Dated: _____
Document # (if appropriate): ___ INV 03-01 _____
Page #(s): ___5_____
Section #: ___15_____
Text to reference: _____
Five replicate tanks of each concentration will be continually exposed for 1, 2, 5, and 10 days

---

Change in Document: _____
_____ – Invertebrates were exposed for 1-5 days, the 10-day exposure was not carried out.

Justification and Impact on Study: _____
Preliminary data suggested that steady state was reached in less than 5 days, thus the 10- day exposure was deemed unnecessary. This did not significantly impact the study.

Submitted by: Signature: _____ Date: 3/26/04

Authorized by: Study Director: _____ Date: 3/26/04

Received by: Quality Assurance Unit: _____ Date: 3/26/04

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

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Check One: _____ Amendment _x___Deviation _____ Addendums

Document Reference Information
Check One: _x___ Protocol _____ SOP _____ Other _____
Title: _____ Perchlorate Uptake in Invertebrates
Dated: _____
Document # (if appropriate): ___ INV 03-01 _____
Page #(s): ___6_____
Section #: ___15_____
Text to reference: _____
Labeling: samples will be labeled with a unique ID number according to the following scheme:

LP (laboratory perchlorate exposure)– species (2 letter abbreviation)- sample number. Species abbreviations will by SN for ramshorn snails and BW for blackworms

E.g., LP- SN- 0001- is the liver sample from snail sample # 0001.

Change in Document: _____
_____ Labeling: samples were labeled with a unique ID number according to the following scheme:

Species (2 letter abbreviation) – trial number - exposure duration (days)- sample number. Species abbreviations will by HS for ramshorn snails (*Heliosoma*) and LV for blackworms

E.g., LV-1 –2 – 3 blackworm sample #3, 2-day exposure, trial # 1.

Justification and Impact on Study: _____
The labeling system was changed to facilitate identification of samples.

---

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

Submitted by: Signature: _____ Date: 3/26/04

Authorized by: Study Director: _____ Date: 3/26/04

Received by: Quality Assurance Unit: _____ Date: 3/26/04

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Form No. 014 Rev. 3.06/00
Project No.: ___T9700.11_
*Change No: _____3_____
Page: __1___ of ___1__

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment _x___Deviation ____ Addendums

Document Reference Information
Check One: _x___ Protocol ____ SOP ____ Other _____
Title: ____ Perchlorate Uptake in Invertebrates
Dated: _____
Document # (if appropriate): ___ INV 03-01 _____
Page #(s): ___6_____
Section #: ___15_____
Text to reference: _____
"PROPOSED STATISTICAL METHODS:
To statistically determine the differences between treatments in terms of histological endpoints, thyroid hormone or perchlorate body concentrations, 2-way ANOVA will be used to determine effects of concentration and time of exposure. Correlation and regression analysis may also be used to determine the relationship between response (body burden, thyroid function) vs. dose or vs. time. "

Change in Document: _____
_____ All data were checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances wad checked using Bartlett's or Lavine's test. Comparisons between treatments was accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons.

Justification and Impact on Study: _____
The statistical methodologies listed in the protocol were incorrect and changed to reflect correct analyses.This did not significantly impact the study.

Submitted by: Signature: _____ Date: 3/26/04

Authorized by: Study Director: _____ Date: 3/26/04

Received by: Quality Assurance Unit: _____ Date: 3/26/04

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment _x___Deviation ____ Addendums

Document Reference Information
Check One: _x___ Protocol ____ SOP ____ Other _____
Title: ____ Perchlorate Uptake in Invertebrates
Dated: _____
Document # (if appropriate): ___ INV 03-01 _____
Page #(s): ___7_____
Section #: ___16_____
Text to reference: _____
"PROPOSED STATISTICAL METHODS:
To statistically determine the differences between treatments in terms of histological endpoints, thyroid hormone or perchlorate body concentrations, 2-way ANOVA will be used to determine effects of concentration and time of exposure. Correlation and regression analysis may also be used to determine the relationship between response (body burden, thyroid function) vs. dose or vs. time. "

Change in Document: _____
_____ All data were checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances wad checked using Bartlett's or Lavine's test. Comparisons between treatments was accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons.

Justification and Impact on Study: _____
The statistical methodologies listed in the protocol were incorrect and changed to reflect correct analyses.This did not significantly impact the study.

Submitted by: Signature: _____ Date: 3/26/04

Authorized by: Study Director: _____ Date: 3/26/04

Received by: Quality Assurance Unit: _____ Date: 3/26/04

* Sequentially numbered in order of the date that the change is effective

29 MAR 2004

Immune Responses to Perchlorate in Native Amphibians

STUDY NUMBER:	SPEA-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	July 1st, 2003
RESEARCH COMPLETION:	Feb 29th, 2004

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Scott T. McMurry

3/29/04
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 29, 2004
Date

1.0 DESCRIPTIVE STUDY TITLE:

Immunotoxicity, as measured by lymphocyte proliferation, white blood cell counts, splenocytes, and spleen weight of sodium perchlorate to wild-caught tadpoles (*Spea bombifrons and Spea multiplicata*) exposed in the lab.

2.0 Study Number

SPEA-03-01

3.0 Sponsor

Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 Testing Facility Name and Address

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 Proposed Experiment Start and Termination Dates

Start: July 1st, 2003
Termination: Feb 29th, 2004

6.0 Key Personnel

Dr. Scott T. McMurry, Study Director
Mrs. Amy R. Hensley, Co-Investigator
Ms. Mary B. Gutierrez, Co-Investigator
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Testing Facility Management

7.0 Study Objectives/Purpose

To evaluate the immune response effects of sodium perchlorate exposure to native species of toads (*Spea bombifrons* and *Spea multiplicata*).

8.0 Study Summary

Spea tadpoles were caught in a Lubbock-area playa lake and brought into the lab. In the lab, the tadpoles were divided into 4 groups and their water was dosed with 0, 20 ppb, 150 ppb, or 100 ppm sodium perchlorate. As the tadpoles metamorphed they were removed from the water and put into tanks containing soil, ending their exposure. The toads were then allowed to grow to a size that was large enough to provide enough spleen cells to perform the lymphycyte proliferation assay. In two batches, on consecutive days, the toads were euthanized and tested for immune

system effects. Information such as spleen weight, spleen cellularity, red blood cells, white blood cells, animal weight, splenocytes responsiveness to a mitogen, and animal mass were recorded to give insight into the animals' immune health.

9.0 Test Materials

Test Chemical name:	Sodium perchlorate
CAS number:	7601-89-0
Characterization:	minimum 99%
Source:	Sigma Aldrich

10.0 Justification of Test System

The plains spadefoot toad, *Spea bombifrons,* and the New Mexico spadefoot toad, *Spea multiplicata,* are both native species to Lubbock-area playa lakes (Behler et al. 1979). After hibernating through the winter, the toads awaken with the arrival of spring rain showers. This is followed by an intense period of breeding. Their tadpoles are abundant in playas for a couple of weeks during the summer months. Because these toads or closely related toads inhabit large portions of the Great Plains of the United States, they are likely to inhabit areas that are known to be contaminated with perchlorate. This contamination has been found in numerous states, including Texas, Arizona, Nevada, Utah, Pennsylvania, New York, and others. Even if these species do not live in these areas, it is likely that very similar species of toads do inhabit contaminated areas. Using the New Mexico spade foot toad and the plains spadefoot will allow for the assessment of the risk of perchlorate exposure to the immune systems of native amphibian species.

Additionally, amphibians are likely to be very susceptible to impairment from perchlorate exposure. Perchlorate is a potent thyroid inhibitor of both mammals and amphibians (Wolff 1998). Because amphibian's metamorphosis is largely regulated by thyroid hormones (Shi 2000), perchlorate exposure has been found to inhibit or prevent metamorphosis of tadpoles (Goleman et al. 2001). Amphibians that do not go through metamorphosis are at a severe disadvantage; likely to succumb to predators, illness, or the upcoming stresses of winter.

As the metamorphosis of amphibians is affected by perchlorate, their immune systems are indirectly affected. The immune system undergoes extensive reorganization during metamorphosis, theoretically to prevent an autoimmune response to the newly forming, adult-specific molecules (Rollins-Smith et al. 1992). With the inhibition of metamorphosis, the reorganization processes have the potential to be adversely affected. Perchlorate has been found to be an immunotoxic chemical in amphibians as its effects on metamorphosis indirectly affect immunity (Rollins-Smith 1992). When metamorphosis and possibly the immune system are inhibited as with perchlorate exposure there is significant risk to the recruitment of adults and the maintenance of a viable amphibian population.

11.0 Test Animals

Species:	New Mexico spadefoot toad (*Spea multiplicata*) and the Plains spadefoot toad (*Spea bombifrons*)
Age:	tadpoles when caught, raised to adulthood in the lab

Number: 300 *Spea*
Source: playa lakes of the South Plains area surrounding Lubbock

12.0 Procedure for Identifying the Test System

When the tadpoles were caught from a playa lake they were placed into buckets that were labeled with playa name, playa location, date of capture. Once in the lab, the toads were randomly divided into separate aquaria, 25 to a tank. Each aquarium was labeled with all pertinent information (AUP number, species, emergency contact information, testing substance, investigator, project number, date of capture, dosing group).

13.0 Experimental Design Including Bias Control

The immunocompetence of metamorphed toads that had been exposed to different concentrations of perchlorate (including a control group) as tadpoles was assessed using a lymphocyte proliferation assay, white blood cell counts, splenocyte number, and spleen weight. Tadpoles captured in the wild (TIEHH SOP AQ-3-05) were brought in the lab and randomly assigned to dose groups. Each dose group received a different concentration of perchlorate (20 ppb, 150 ppb, 100 ppm, 0 ppm), one being a control group. Each dose was tested in triplicate (3 identical tanks) and in each tank there were 25 tadpoles. The groups were maintained in their exposure concentration until they had completed metamorphosis. They were then sacrificed so as to assess them for immune effects. Lymphocyte proliferation, spleen cellularity, white blood cell counts, and spleen weight were used to assess the amphibians' immune health. After the groups had completed metamorphosis and had reached an adequate size, they were euthanized. The immunoassays could only be performed once the amphibians had completed metamorphosis and had grown to a size that was large enough for their spleens to provide sufficient splenocytes.

14.0 Methods

14.1 Test System acquisition, quarantine, acclimation

300 tadpoles (*Spea multiplicata* and *Spea bombifrons)* were caught in a Lubbock-area playa lake with the use of nets attached to long poles. When caught, the tadpoles were placed into 5 gallon buckets containing playa water that were labeled with the location in which they were found. The buckets were transported to the lab at TIEHH in the back of a truck where they were well secured with rope and shaded from too much sunlight. When brought to the lab at TIEHH, the tadpoles were divided randomly into dose groups and placed into their tanks. For 3 days before testing begins, the amphibians were allowed to acclimatize. The tadpoles were cared for following the *Xenopus laevis* husbandry (TIEHH SOP AQ-1-06) and fed rabbit chow.

14.2 Test Material Application

Rates/concentrations, frequency, route: The tadpoles are aquatic, so they were housed in water containing 20 ppb, 150 ppb, 100 ppm, or 0 ppm sodium

perchlorate starting 3 days after their capture and ending when they completed metamorphosis.

Justification for Exposure Route: This exposure route mimics the manner in which they would be exposed to perchlorate in the wild.

Exposure Verification: Samples of the water were taken approximately every week to verify that the needed concentration of perchlorate was present. Ion chromatography was used to measure the level of perchlorate (TIEHH SOP GW-02-03)

14.3 Test System Observation

Tadpoles were monitored daily. Metamorphs were removed and housed in aquaria containing soil and a dish of water. These metamorphs were fed mealworms.

14.4 Animal Sacrifice and Sample Collections

The toads were euthanized in a MS-222 solution as in TIEHH SOP AQ-1-03 and then weighed. The toads were then decapitated and whole blood samples were collected from the trunk of the toad. Their spleens were removed and spleen weight recorded, aseptically. Each spleen was used immediately in the immunoassay, so no storage was needed.

14.5 Endpoint Analysis

The general study endpoint was to detect a difference in the immune responses of perchlorate treated tadpoles and non-exposed tadpoles. The lymphocyte proliferation assay measures lymphocyte's ability to proliferate as they would in response to cytokines signaling that an infection is present. White blood cell counts, spleen weight, and spleen cellularity also give insight into the status of the immune system's cells.

Lymphocyte Proliferation Assay

All procedures were done under sterile conditions. A spleen was obtained aseptically and its mass recorded. The splenocytes was dispersed into amphibian phosphate buffered saline (APBS) with a sterile glass homogenizer. The cell suspension was then centrifuged at 1100 rpm for 7 minutes at 10°C and the supernatant discarded. The pellet was resuspended in APBS (HPLC grade water, sodium chloride, sodium phosphate, and potassium phosphate). These two steps were repeated twice more and then the number of cells were counted in a 1:2 dilution of Trypan blue. Only viable white blood cells were recorded. After this the cell suspension was centrifuged and brought up to the proper volume in Leibovitz-15 media (L-15) so that the concentration was $5x10^4$ white blood cells / 100 µl L-15. The L-15 was supplemented with hepes, antibiotics, L-glutamine, 10% fetal bovine serum, and Na pyruvate and brought to amphibian osmolality (200 mOsm). 100 µl of the cell solution was placed into each well of a 96 well plate. The mitogen was

lymphpolysaccharide (LPS) as it had been found to be most effect at stimulating proliferation in *Spea*. Stock solutions of the appropriate concentration of mitogen in L-15 were prepared ahead of time. The concentrations used were 20, 10, 5, and 2.5 µg / ml media. Priority was given to 20 µg / ml media for each toad as there were often times not enough splenocytes to test multiple concentrations of mitogen. 20 µl of the appropriate mitogen was added to each appropriate well. A minimum of duplicates was used for each concentration of mitogen, triplicates when possible. One set did not receive any mitogen (cell suspension only) to provide blank wells to assess background proliferation. The well plates were then put into an incubator with 5% CO_2 at 27°C for 48 hours. Pulsing included adding 20 µl of a 50 µCi/ml solution of ^{3}H-thymidine to each well (1 µCi / well). Then, after a 24-hour incubation, the cells were harvested using a Brandel® cell harvester. The level of radioactivity was measured with a scintillation counter (count time = 1 min/sample). As a tool for analyzing the results of the proliferation assay a stimulation index (SI) was calculated. The SI is equals to the cpm of the wells that received some concentration of LPS divided by the cpm of the wells that did not receive any mitogen, the blanks.

15.0 Results

In July 2003, 300 *Spea* tadpoles were captured from a Lubbock-area playa lake and in the subsequent months of exposure and development there were many mortalities due to factors such as the emergence of cannibal morphs among the tadpoles, water quality issues, and other unknown reasons. The toads were susceptible both before and after metamorphosis. The final sample size for the control group was 13, 1 in the 20 ppb dose group, 9 in the 150 ppb dose group, and 6 in the 100 ppm dose group. The mortality was not dependent on the dose. The 20 ppb dose group was removed from further analysis because it consisted of only one animal.

Table 1. Nominal and analytically detected concentrations of perchlorate in the tadpole exposure media.

	Nominal Concen (ppb)	Detected Concen (ppb)[a]
Control	0	11
150 ppb	150	263
100 ppm	100,000	96,553

[a] The mean perchlorate concentration in the water samples taken from the tadpoles' tanks during exposure.

Although care was taken to dose the tadpoles with specific concentrations of perchlorate, there was some variation from the nominal concentrations (Table 1). In two out of three cases the perchlorate concentrations were found to be slightly greater than expected. With concentrations of 11 ppb, 263 ppb, and 97 ppm rather than 0, 150 ppb, and 100 ppm, the analytically detected concentrations were essentially equivalent to the nominal concentrations. These detected concentrations were comprised of a low, medium, and a very

high concentration, achieving the range that was intended. From this point on the nominal concentrations will be referred to although they differed somewhat from what was found.

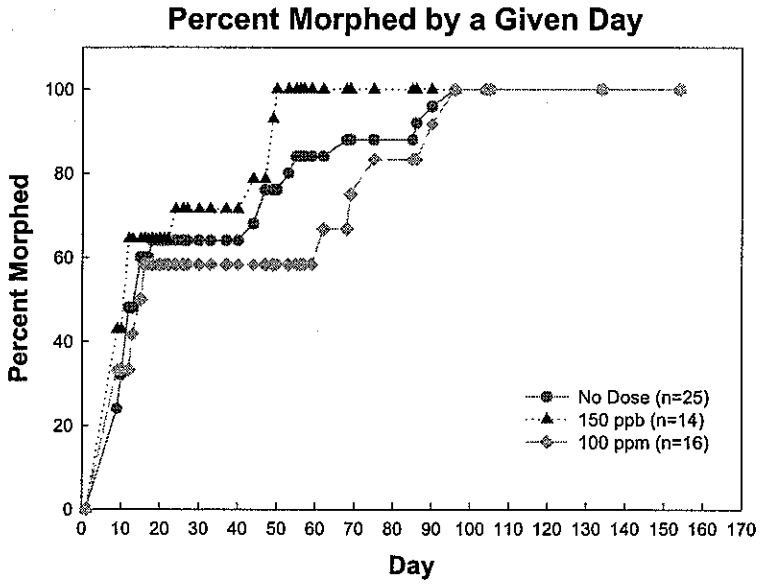

Fig. 1 Percent of the number of tadpoles that lived through metamorphosis that had metamorphed by a given day.

Metamorphosis rates appear to have been affected by the perchlorate (Fig. 1). The tadpoles exposed to 100 ppm metamorphed the slowest out of the three groups followed by the control group and then the 150 ppb dose group. Statistical analysis has not yet been performed on these metamorphosis rates.

Table 2. Mean (±SEM) values for morphometric and immunological indices in spadefoot toads exposed to different concentrations of sodium perchlorate as tadpoles.

Variable	Control		150 ppb		100,000 ppb	
	n	mean	n	mean	n	mean
Body Weight (g)	13	2.7 ± 0.34	9	3.5 ± 0.38	6	3.3 ± 0.85
SVL[a] (mm)	13	27 ± 0.9	9	29 ± 1.2	6	29 ± 2.3
WL[b] (mm)	13	52 ± 1.7	9	55 ± 1.9	6	55 ± 4.4
Spleen Weight (mg)	13	10.23 ± 2.581	9	8.21 ± 1.412	6	7.81 ± 1.833
Splenocyte Number ($x10^3$)	13	394.2 ± 230.95	9	145.8 ± 45.64	6	200.7 ± 118.02
Blank (cpm)	12	240.8 ± 34.34	8	435.3 ± 167.60	4	248.3 ± 131.96
20µg/ml LPS (SI[c])	7	3.9 ± 1.21	4	4.0 ± 1.71	2	4.9 ± 1.83
WBC ($x10^3$)	12	7.65 ± 0.992	8	8.57 ± 1.625	6	8.06 ± 1.709
RBC ($x10^6$)	12	0.99 ± 0.063	9	0.87 ± 0.059	6	1.04 ± 0.067
PCV (%)	5	29 ± 3.2	4	25 ± 2.4	5	33 ± 2.7

[a]SVL = snout vent length
[b]WL = whole toad length
[c]SI = stimulation index

Table 3. Results of non-parametric analysis of the morphometric and immunological indices ($p \leq 0.05$ was considered to be significant).

Variable	F Statistic	P-value
Body Weight (g)	1.54	0.2340
SVL[a] (mm)	0.79	0.4660
WL[b] (mm)	0.88	0.4271
Spleen Weight (mg)	0.05	0.9490
Splenocyte Number ($x10^3$)	0.07	0.9309
Blank (cpm)	0.69	0.5130
20 µg/ml LPS (SI[c])	0.15	0.8621
WBC ($x10^3$)	0.16	0.8531
RBC ($x10^6$)	2.02	0.1542
PCV (%)	1.36	0.2974

[a]SVL = snout vent length
[b]WL = whole toad length
[c]SI = stimulation index

There were no statistically significant differences found between the dose groups. P-values of less than 0.05 were considered to be significant. No differences were evident either with preliminary evaluation of the means or after statistical analysis among the morphometric indices; body weight, snout-vent length, whole length, or spleen weight (table 3). It did appear, though, that there was a possible difference between dose group means for splenocyte numbers (394, 148, and 200 $x10^3$ cells) but statistical analysis showed no significant difference. This lack of significance is explained by the fact that the sample sizes were small (13, 9, and 4) with large standard errors (231, 46, and 118 cpm). From the lymphocyte proliferation assay differences were also suspected after looking at the means of the blank wells (241, 435, and 248 cpm), however, these were also not significant as the sample sizes were small (12, 8, and 4) while standard errors were high (34, 168, and 132). The wells to which 20µg/ml of LPS were added exhibited no differences as did each of the hematological data (RBC, WBC, and PCV). Among all of the endpoints, a few possible differences were found when the means were calculated and evaluated, however, the small sample sizes combined with large variability masked any such possible differences.

16.0 Discussion

In this experiment there were interesting differences between what was expected and what was found. In several cases there were differences that appeared when the means were evaluated but were so masked by variability and small sample sizes that no significant differences were found in the statistical analysis. This draws attention to some of the indices, perhaps indicating that there were effects from the perchlorate exposure, but does not allow for statistically sound conclusions of effects from the exposure to be made. It should be noted that all expectations were drawn from research done on *Xenopus* or *Rana* exposed to perchlorate. These species' effects from perchlorate exposure may not necessarily be seen in *Spea* as it was assumed in this research.

The metamorphosis rates of the tadpole dose groups did not entirely fit predicted patterns (fig. 1). As perchlorate inhibits metamorphosis in amphibians (Goleman et al., 2002) due to its inhibitory effects on the thyroid hormones that regulate metamorphosis (Galton, 1988), the higher concentrations of perchlorate were expected to delay metamorphosis. Goleman et al. also found the effects of perchlorate to be dose-dependent so supporting expectations of greater metamorphosis inhibition with greater doses of perchlorate. Therefore, it was somewhat unexpected for the 150 ppm dose group to metamorph the quickest. The control group metamorphed slower than the 150 ppb group, but quicker than the 100 ppm group. So, the 100 ppm and the control group exhibit the expected pattern with respect to each other while the 150 ppb's swift metamorphosis is inexplicable.

Morphometric indices such as animal mass, snout-vent length, whole length, and spleen weight were not necessarily expected to be affected by the perchlorate exposure. This expectation stems from research that found *Xenopus* snout-vent lengths of exposed and unexposed animals to converge given enough recovery time (Goleman et al., 2002). As there were several months between the exposure and the analysis, there was ample time for differences in these indices to diminish. Preliminary evaluation of the means did not draw attention to possible significant differences in any of these endpoints, so there is probably no masking occurring. This leaves two possible explanations for why there were no morphometric differences found between dose groups. There may not have been any differences at any point in the experiment due to perchlorate exposure or any differences that existed during perchlorate exposure diminished after exposure was terminated.

As a result of the thyroidal effects of perchlorate, immune effects have been found in amphibians dosed with perchlorate (Kinney et al. 1996, Rollins-Smith and Blair 1990, Miranda and Dezi 1997, Kanki and Wakahara 2000, and Rollins-Smith et al. 1992). Endpoints such as splenocyte number, background proliferation of splenocytes, splenocyte response to LPS, and number of WBC's in the blood are indicators of immune effects from exposure (Gilbertson et al., 2003, Christin et al., 2003). In this experiment no immune effects from exposure to perchlorate were detected. No statistical significance was found in any case. Although there may be differences that were being masked, there is also a possibility that the immune system had recovered from any effects that were present while exposure was occurring. If wild toads were exposed with perchlorate as tadpoles and managed to metamorph, these possible immune effects may not significantly decrease their fitness if they are able to recover as adults that live mostly out of the perchlorate contaminated water. That is if they did not succumb to an illness before they are able to recover.

Overall, there were no significant differences in any endpoint tested between the dose groups of toads. There are possibly no effects from the exposure seen in *Spea.* There is also a strong possibility that small sample sizes and extensive variability are concealing some effects. As immune effects have the potential to drastically reduce the survivorability of an organism, it is a possibility that should not be disregarded and perhaps studied further.

17.0 References

Behler, JL. 1979. National Audobon Society Field Guide to North American Reptiles and Amphibians. Alfred A Knopf, New York.

Christin MS, Gendron AD, Brousseau P, Menard L, Marcogliese DJ, Cyr D, Ruby S, Fournier M. Effects of agricultural pesticides on the immune system of *Rana pipiens* and on its resistance to parasitic infection. Environmental Toxicology and Chemistry 22(5):1127-1133.

Galton, VA. 1988. The role of thyroid hormone on amphibian development. American Zoology 28:309-318.

Gilbertson MK, Haffner GD, Drouillard KG, Albert A, Dixon B. 2003. Immunosuppression in the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. Environmental Toxicology and Chemistry 22(1):101-110.

Goleman WL, Uruidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environmental Toxicology and Chemistry 21:424-430.
Kinney et al. 1996

Kanki K, Wakahara M. 2000. Spatio-temporal expression of TSH beta and FSH beta genes in normally metamorphosing, metamorphosed, and metamorphosis-arrested Hynobius retardatus. General and Comparative Endocrinology. 119(3):276-86.

Miranda LA, Pisano A, Casco V. 1996. Ultrastructural study on thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20(2):147-153.

Rollins-Smith LA, Blair P. 1990. Expression of class II major histocompatibility complex antigens on adult t-cells in *Xenopus* is metamorphosis-dependent. Developmental Immunology 1:97-104.

Rollins-Smith LA, Blair PJ, Davis AT. 1992. Thymus ontogeny in frogs: t-cell renewal at metamorphosis. Developmental Immunology 2:207-213.

Shi, Yun-Bo. 2000. Amphibian metamorphosis. John Wiley and Sons, Inc. New York, New York.

Wolff J. 1998. Perchlorate and the thyroid gland. Pharmacological Reviews 50(1):89-105.

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Immune System Responses to Perchlorate Exposure in Native Species of Toads, *Spea bombifrons* and *Spea multiplicata*

STUDY/PROTOCOL NUMBER: SPEA-03-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY:

Name/Address: The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall
Director, TIEHH

Study Director: Dr. Scott T. McMurry

PROPOSED EXPERIMENTAL
START DATE: JULY 1, 2003

1. **DESCRIPTIVE STUDY TITLE:**
Immunotoxicity, as measured by lymphocyte proliferation and white blood cell counts, of sodium perchlorate to wild-caught tadpoles (*Spea bombifrons and Spea multiplicata*) exposed in the lab

2. **STUDY NUMBER**: SPEA-03-01

3. **SPONSOR:** Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. **TESTING FACILITY NAME & ADDRESS:**

The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: July 01, 2003
Termination Date: March 31, 2004

6. **KEY PERSONNEL:**
Dr. Scott T. McMurry, Study Director
Mrs. Amy R. Hensley, Co-Investigator
Ms. Mary B. Gutierrez, Co-Investigator
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Testing Facility Management

7. **DATED SIGNATURES:**

Scott McMurry 3/11/04 Dr. Scott T. McMurry
Study Director

Ronald J. Kendall 3/15/04 Dr. Ron Kendall
Testing Facility Management

3-11-04 Mr. Ryan Bounds
Quality Assurance Manager

3-12-04 Dr. Todd Anderson
Asst. Dir. For Science

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in the spirit of Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to TIEHH and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. Scott T. McMurry
TIEHH
Box 41163
Lubbock, TX 79409-1163

9. STUDY OBJECTIVES / PURPOSE:

To evaluate the immune response effects of sodium perchlorate exposure to native species of toads (*Spea bombifrons* and *Spea multiplicata*)

10. TEST MATERIALS:

Test Chemical name:	Sodium perchlorate
CAS number:	7601-89-0
Characterization:	minimum 99%
Source:	Sigma Aldrich

11. JUSTIFICATION OF TEST SYSTEM

The plains spadefoot toad, *Spea bombifrons,* and the New Mexico spadefoot toad, *Spea multiplicata,* are both native species to Lubbock-area playa lakes (Behler et al. 1979). After hibernating through the winter, the toads awaken with the arrival of spring rain showers. This is followed by an intense period of breeding. Their tadpoles are abundant in playas for a couple of weeks during the summer months. Because these toads or closely related toads inhabit large portions of the Great Plains of the United States, they are likely to inhabit areas that are known to be contaminated with perchlorate. This contamination has been found in numerous states, including Texas, Arizona, Nevada, Utah,

Pennsylvania, New York, and others. Even if these species do not live in these areas, it is likely that very similar species of toads do inhabit contaminated areas. Using the New Mexico spade foot toad and the plains spadefoot will allow for the assessment of the risk of perchlorate exposure to the immune systems of native amphibian species.

Additionally, amphibians are considered the most susceptible to impairment from perchlorate exposure. Perchlorate is a potent thyroid inhibitor of both mammals and amphibians (Wolff 1998). Because amphibian's metamorphosis is largely regulated by thyroid hormones (Shi 2000), perchlorate exposure has been found to inhibit or prevent metamorphosis of tadpoles (Goleman et al. 2001). Amphibians that do not go through metamorphosis are at a severe disadvantage; likely to succumb to predators, illness, or the upcoming stresses of winter.

As the metamorphosis of amphibians is affected by perchlorate, their immune systems are indirectly affected. The immune system undergoes extensive reorganization during metamorphosis, theoretically to prevent an autoimmune response to the newly forming, adult-specific molecules (Rollins-Smith et al. 1992). With the inhibition of metamorphosis, the reorganization processes have the potential to be adversely affected. Perchlorate has been found to be an immunotoxic chemical in amphibians as its effects on metamorphosis indirectly affect immunity (Rollins-Smith 1992). When metamorphosis and possibly the immune system are inhibited as with perchlorate exposure there is significant risk to the recruitment of adults and the maintenance of a viable amphibian population.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system)**:**

Species: New Mexico spadefoot toad (*Spea multiplicata*) and the Plains spadefoot toad (*Spea bombifrons*)

Age: tadpoles when caught, raised to adulthood in the lab

Number: 300 *Spea*

Source: playa lakes of the South Plains area surrounding Lubbock

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

When the tadpoles are caught from a playa lake they will be placed into buckets that are labeled (playa name, playa location, date of capture). Once in the lab, the toads will be randomly divided into separate aquaria, 25 to a tank. Each aquarium will be labeled with all pertinent information (AUP number, species, emergency contact information, testing substance, investigator, project number, date of capture, dosing group).

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

The immunocompetence of metamorphed toads that have been exposed to different concentrations of perchlorate (including a 0 concentration control group) as tadpoles will be assessed using a lymphocyte proliferation assay and white blood cell counts. Tadpoles captured in the wild (TIEHH SOP AQ-3-05) will be brought in the lab and randomly assigned to dose groups. Each dose group will receive a different concentration of perchlorate (20 ppb, 150 ppb, 100 ppm, 0 ppm), one being a control group. The groups will be maintained in their exposure concentration until after they have completed metamorphosis. Then, they will be sacrificed so as to run the immunoassay on them. Lymphocyte proliferation, spleen cellularity, white blood cell counts, and spleen weight will be used to assess the amphibians' immune health. Exposure to perchlorate should inhibit metamorphosis, so metamorphosis will proceed, but at a slower pace. After the groups have completed metamorphosis, they will be euthanized. The immunoassays can only be performed once the amphibians have completed metamorphosis and have grown to a size that is large enough for their spleens to provide sufficient splenocytes.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

300 tadpoles (*Spea multiplicata* and *Spea bombifrons)* will be caught in Lubbock-area playa lakes with the use of nets attached to long poles. When caught, the tadpoles will be placed into 5 gallon buckets containing water from the playa that are labeled with the location in which they were found. The buckets will be transported to the lab at TIEHH in the back of a truck where they will be well secured with rope and shaded from too much sunlight. When brought to the lab at TIEHH, the tadpoles will be divided randomly into dose groups and placed into tanks. For 3 days before testing begins, the amphibians will be allowed to acclimatize. The tadpoles will be cared for following the *Xenopus laevis* husbandry (TIEHH SOP AQ-1-06) and fed fresh spinach (they are vegetarian) every two days.

15.2 Test Material Application

Rates/concentrations, frequency, and route: The tadpoles are aquatic, so they will be housed in water containing 20 ppb, 150 ppb, 100 ppm, or 0 ppm sodium perchlorate starting 3 days after their capture and ending at the appropriate life stage.

Justification for Exposure Route: This exposure route mimics the manner in which they would be exposed to perchlorate in the wild.

Exposure Verification: Samples of the water will be taken every week to verify that the needed concentration of perchlorate is present. Ion chromatography will be used to measure the level of perchlorate (TIEHH SOP GW-02-03)

15.3 Test System Observation

Tadpoles will be monitored daily. Metamorphs will be removed and housed in aquaria containing soil and a dish of water. These metamorphs will be fed mealworms every two days.

15.4 Animal Sacrifice and Sample Collections

The toads will be euthanized in a MS-222 solution (TIEHH SOP AQ-1-03) and then weighed. Their spleens will be removed and spleen weight recorded, aseptically. Each spleen will be used immediately in the immunoassay, so no storage is needed. Whole blood samples will be collected from the heart of the toad. The liver will also be removed and weighed for possible metals residue analysis at a later date in a separate study. The liver and carcass will be frozen together in the meantime.

15.5 Endpoint Analysis

The main study endpoint is to detect a difference in the immune responses of perchlorate treated tadpoles and non-exposed tadpoles. The lymphocyte proliferation assay measures lymphocytes ability to proliferate as they would in response to cytokines signaling that an infection is present. White blood cell counts, spleen weight, and spleen cellularity will each give insight into the status of the immune system's cells.

16. PROPOSED STATISTICAL METHODS

We will use standard testing methods, to include regression and analysis of variance, with a significance value of 5%, using the JMP software by SAS.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include dosing schedule, metamorphosis rate, when water samples are taken, number of animals that have metamorphed and been moved to soil aquaria, feeding schedule, deaths, and any abnormal behavior.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- Whole animal weight
- Animal length (snout-vent and whole body)
- Spleen weight
- Number of splenocytes
- Level of lymphocyte proliferation in response to mitogens
- White blood cell counts

Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before March 31st, 2004. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Behler, JL. 1979. National Audobon Society Field Guide to North American Reptiles and Amphibians. Alfred A Knopf, New York.

Goleman WL, Uruidi LJ, Anderson TA, Smith EE, Kendall RJ, Carr JA. 2002. Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environmental Toxicology and Chemistry 21:424-430.

Rollins-Smith LA, Blair PJ, Davis AT. 1992. Thymus ontogeny in frogs: t-cell renewal at metamorphosis. Developmental Immunology 2:207-213.

Shi, Yun-Bo. 2000. Amphibian metamorphosis. John Wiley and Sons, Inc. New York, New York.

Wolff J. 1998. Perchlorate and the thyroid gland. Pharmacological Reviews 50(1):89-105.

A FINAL REPORT

ENTITLED

PECHLORATE IN INVERTEBRATES, PERIPHYTON, AND DETRITUS AT THE NAVAL WEAPONS INDUSTRIAL RESERVE PLANT, MCLENNAN COUNTY, TEXAS

STUDY NUMBER:	AQUA 03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	02/07/2003
RESEARCH COMPLETION:	12/31/2003

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GOOD LABORATORIES PRACTICES STATEMENT

Project AQUA 03-01, entitled " Perchlorate in Invertebrates, Periphyton, and Detritus at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/25/03
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

Submitted By:

Ryan Bounds
Quality Assurance Manager

3-25-04
Date

1. DESCRIPTIVE STUDY TITLE:
Perchlorate in Invertebrates, Periphyton, and Detritus at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

2. STUDY NUMBER:
AQUA 03-01

3. SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. EXPERIMENTAL START & TERMINATION DATES:
Start: 03/28/2003
Termination: 12/31/2003

6. KEY PERSONNEL:
Ron Kendall, Principal Investigator
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Richard Hanson, Quality Assurance Officer
Les McDaniel, Graduate Student
Fujun Liu, Graduate Student

7. STUDY SUMMARY:
In April and June, 2003, samples of water, periphyton (filamentous green algae), detritus, and invertebrates (snails, crayfish, clams, insects, and insect larvae) were collected from 5 creeks in and around the Texas Naval Weapons Industrial Reserve Plant (NWIRP). Harris Creek, North Fork South Bosque, South Fork South Bosque and Station Creek all originated on the NWIRP. Wasp creek does not originate on the NWIRP but a portion of the creek runs through it. Perchlorate was detected in water samples all creeks originating on the NWIRP. In the April 2003 sampling trip, perchlorate was detected in several periphyton samples, one detritus sample, and in only one invertebrate sample (snail). In the June 2003 sampling trip, perchlorate was detected in periphyton and several invertebrates. In many instances, the concentrations of perchlorate in periphyton and invertebrates was greater than that in the water, and perchlorate was often detected in these samples when it was not detected in the

water.

8. STUDY OBJECTIVES / PURPOSE:

To determine comparative body burdens of perchlorate in detritus, periphyton, and invertebrates collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP).

9. TEST MATERIALS:

Test Chemical: Perchlorate anion
CAS Number: 7601-89-9
Characterization: Determination of concentration in environmental samples
Source: Wastewater effluent discharge, groundwater seepage, runoff from or percolation through contaminated soil.

10. JUSTIFICATION OF TEST SYSTEM:

Preliminary surveys of NWIRP have revealed that measurable levels of ammonium perchlorate have been found in surface waters of aquatic systems (i.e., streams, lakes, and rivers) within and adjacent to NWIRP. Comparative analysis of perchlorate accumulation in fish and water from field-collected specimens indicates that perchlorate levels may be higher in fish than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish. This suggests that fish in the field are being exposed by some route other than direct absorption from the water. Possibly fish are being exposed through the food chain. In the stream ecosystem that have been studied so far, there is little phytoplankton, unlike lakes and larger rivers. Thus the primary productivity ("bottom of the food chain") comes from periphyton (algae film growing on rocks and other solid objects) or introduction of detritus (dead and decaying leaf litter) from terrestrial sources, and the associated saprobes (decomposers: bacteria and fungi) that grow on the detritus. Thus, one possible route of exposure of the fish is via accumulation of perchlorate by the periphyton or saprobes. Also, previous research has found that perchlorate accumulates in terrestrial plants, and most detritus in stream systems comes from fallen leaves of terrestrial plants in the riparian zone (i.e. streamside).
In addition, many fish in stream habitats, especially the ones that have been analyzed so far, feed on aquatic invertebrates. Uptake and possible accumulation from the water has not yet been addressed in invertebrates. Many invertebrates also feed upon detritus, and so could accumulate perchlorate from the detritus, which could then be passed on to fish, but the degree to which invertebrates accumulate perchlorate is currently unknown.

The uptake and bioaccumulation of perchlorate in invertebrates, periphyton, and detritus at perchlorate-contaminated sites has not been studied to date. There is also little information related to the possible uptake of perchlorate via ingestion of contaminated food. Therefore, the purpose of this study is to examine the levels of perchlorate in periphyton, detritus, invertebrates.

11. TEST ANIMALS:

Species: Detritus, periphyton, and invertebrates
Strain: Wild
Age: Various
Number: Maximum of 5 composite samples per species per site.
Source: Captured from natural waters at NWIRP.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of natural waters within NWIRP. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites were selected based on proximity to NWIRP and similarity to NWIRP water bodies, and were found not to contain detectible levels of perchlorate. Each sampling location was labeled with its whole name or a 4-letter abbreviation. Five sites have been identified on NWIRP. Their names (and 4 letter abbreviations) are Harris Creek (HARC), North Fork South Bosque River (NFSB), South Fork South Bosque River (SFSB), Wasp Creek (WASP), and Station Creek (STAC

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Samples were collected from sites of known perchlorate contamination. As many individuals as could be collected were taken from each site, up to 30 individuals per species per site. Each composite sample was composed of specimens collected along an approximately 50 m stretch of stream, and these stretches were located at least 50 m apart from each other.

14. METHODS

14.1. Test System acquisition, quarantine, acclimation

Water Sampling

At each location where samples (detritus, periphyton, & invertebrates) were captured, 60 ml of water was also taken for perchlorate analysis, according to SOP AQ-3-03. Water was collected before collection of samples. Water samples were collected at either end of the section of the stream from which samples were collected, plus at least one sample in between these two points. Samples were taken at least 10 m apart, unless the section of stream to be sampled was less than 10 m long. Water samples were collected in precleaned glass vials (Wheaton), and were collected from just under the water surface. Water samples were stored away from direct sunlight and excessive heat (> 50° C). Before samples were taken, the pH, dissolved oxygen, conductivity, and temperature was measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

14.2. Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.

Frequency: Perchlorate is discharged into surface waters in NWIRP continuously from ground water or wastewater effluent, or is discharged into surface waters after rainfall events via runoff or percolation of rainwater through soil.

Route/Method of Application: Ingestion of absorption of perchlorate from water and natural food items.

Justification for Exposure Route: The animals and plants were exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples were collected for determination of perchlorate concentrations wherever biota samples were collected.

14.3. Test System Observation

At every location from which water samples were taken, the following environmental parameters were evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

14.4. Animal Sacrifice and Sample Collections

Data Recording and Sample Labeling

Prior to processing any samples, they were given a unique ID number and species and weight were recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. According to the SOP IN-3-02, the information to be recorded on labels was the project number and unique ID. We also included on labels the tissue collected, the species, as well as the date of collection. The unique ID, species, date of collection, sex and tissue were also recorded on a sample processing form (e.g. forms 027 "Multiple Fish/Amphibian Dissection/Collection Form" or 182 "Fish Dissection Form"). The unique ID followed the following format: For samples collected from NWIRP, the ID was 4-letter abbreviation of the sampling site)-X-(sample [or composite] number), where X was "D" if the sample was detritus, "P" for periphyton, "C" for crayfish, "SN" for snail, "CL" for clam, or "I" for other invertebrate. If more than one species of invertebrate was collected, it was given an additional designation of A, B, C…eg. IA, IB, IC for "invertebrate A, invertebrate B, invertebrate C," etc.

Perchlorate Analysis

Various species of different trophic levels were collected for perchlorate body burden analysis. Composite samples were placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. After transport to the laboratory, samples were stored in the freezer (temperature -20°C) until analysis. Water samples also were transported back to the laboratory for perchlorate analysis. Once in the laboratory, they were stored in a refrigerator (4°C) until analysis.

14.5. Endpoint Analysis

Perchlorate concentration in the detritus, periphyton, and invertebrate samples were extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in water or extracted from tissues were performed according to SOP AC-2-11 "Analysis of Perchlorate by IC".

15. STATISTICAL METHODS

This project was a survey, rather than hypothesis testing, so no statistical methods were implemented.

16. PROTOCOL CHANGES/REVISIONS:

Initially, fish were collected form NWIRP streams and stomach contents were collected for perchlorate analysis. Unfortunately, because of interfering ions and other compounds, perchlorate could not be quantified in these samples. Resources were then diverted to analysis of other samples. No invertebrates were observed on the detritus, so they were not collected.

17. RESULTS

Several species of invertebrates were collected during April 2003. These included fishing spiders, clams, dragonfly larvae, and snails. Perchlorate was not detected in any of these samples. Perchlorate was found in composite samples of periphyton and one composite sample of detritus (Table 1).

Table 1- Perchlorate concentrations in the water (ng/ml) and detritus or periphyton (ng/g) in samples collected from TNWIRP.

Sampling Site	Sample type	Concentration
Harris Creek	Water	7
	Periphyton	237
South Fork South Bosque	Water	ND*
	Periphyton	ND*
Wasp Creek	Water	ND*
	Detritus	ND*
	Periphyton	10
North Fork South Bosque	Water	6
	Detritus	ND*
Station Creek	Water	8
	Detritus	25.8

*Not detected.

Analysis of the samples collected during June 2003 indicated that perchlorate was consistently detected in crayfish and periphyton, even if perchlorate was not detected in the water. Perchlorate was also detected in other arthropods such as terrestrial isopods (pillbugs) and damselfly (odonate) larvae. Perchlorate was not detected in detritus.

Table 2 - Perchlorate concentrations in the water (ng/ml) and detritus or periphyton (ng/g) in samples collected from TNWIRP.

Sampling site	Sample type	N	Mean +/- SD (Range) perchlorate concentration (ng/g)
South Fork South Bosque	Water	5	ND*
	Crayfish	3	30.8±48.7 (0-87.0)
	Periphyton	5	44.1±79.9 (0-185)
	Diving beetle	1	ND*
	Snail	1	ND*
	Terrestrial isopod (pillbug)	2	74.6±95.6 (7-142)
	Detritus	2	ND*
North Fork South Bosque, downstream site	Water	3	ND*
	Periphyton	3	ND*
	Clam	1	12.7
	Snail	1	ND*
North Fork South Bosque, upstream site	Water	4	5±1.8
	Crayfish	1	ND*
	Periphyton	2	26.2±37.1 (0-52)
	Snail	1	ND*
	Detritus	1	ND*
Wasp Creek	Water	4	ND*
	Damselfly larvae	1	55.3
	Periphyton	3	33.2±44 (1.39-14.8)
	Water Strider	1	16.7
	Snail	1	2.1
	Detritus	1	ND*
Station Creek	Water	4	10±0.4
	Macrophyte	1	ND*
	Periphyton	3	0.5±0.8 (0-1.39)
	Crayfish	2	23.2±15 (13-33.8)
	Clam	1	ND*
	Detritus	1	ND*
Harris	Water	4	ND*
	Periphyton	2	1.4±0.4 (1.1-1.7)
	Detritus	2	ND*

18. DISCUSSION

The present study indicates that perchlorate is taken up by periphyton and invertebrates at NWIRP. Perchlorate concentrations in water invertebrates were similar to those reported at the Longhorn Army Ammunition Plant, Karnack, TX (Smith et al., 2001). Although perchlorate is sporadically found in the detritus, it may not represent a major pathway of exposure, due to the low occurrence of perchlorate in detritus. It would seem that monitoring perchlorate in periphyton is a more reliable indicator of perchlorate exposure in streams than would monitoring water concentrations themselves.

Comparative analysis of perchlorate accumulation in biota and water from field collected specimens indicates that perchlorate levels may be higher in biota than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish (Theodorakis et al., unpublished data). This suggests that fish collected in the field are being exposed by some routes other than direct absorption from the water. One possible route of uptake in fish may be through the food chain. In the stream ecosystems, the primary productivity is due to periphyton (algae film growing on rocks and other solid objects). Therefore, one possible route of exposure of fish is via accumulation of perchlorate by the periphyton. The data presented here support the hypothesis that periphyton may be a source of perchlorate in these contaminated streams. Herbivorous fish, such as stonerollers (*Campostoma anomalum*) may be exposed to perchlorate through this route. The invertebrates also take up perchlorate, although it is uncertain whether they take it up primarily from water or from periphyton. Crayfish seem more prone to accumulate perchlorate above water concentrations than other invertebrates. Terrestrial arthropods such as isopods collected from the waters edge also accumulate perchlorate. This may represent a possible pathway of perchlorate exposure to fish, such as green sunfish (*Lepomis cyanellus*) and largemouth bass (*Micropeterus salmoides*), that regularly feed on crayfish and terrestrial arthropods (terrestrial insects and isopods have been found in the stomachs of green sunfish collected from these streams; pers. obs.). In addition, certain species of wildlife, such as raccoons (*Procyon lotor*), watersnakes (*Natrix spp.*), and herons (*Ardea spp.*) may also feed upon crayfish. Thus, there are possible food-chain pathways for not only transport of perchlorate from the terrestrial to the aquatic compartments, but vice versa as well.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Smith, N.S., Theodorakis, C.W., Anderson, T.A., and Kendall, R.J., 2001. Preliminary assessment of pechlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

21. APPENDICES:

Study Protocol
Changes to Study Documentation

Project No. T9700

A STUDY PROTOCOL

ENTITLED

PECHLORATE IN INVERTEBRATES, PERIPHYTON, DETRITUS, AND FISH STOMACH CONTENTS AT THE NAVAL WEAPONS INDUSTRIAL RESERVE PLANT, MCLENNAN COUNTY, TEXAS

STUDY/PROTOCOL NUMBER: AQUA-03-01

SPONSOR:
US Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

ADMINISTRATOR:
The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, TX 79409-41163

TESTING FACILITY:

Name/Address:
The Institute of Environmental and Human Health
Texas Tech University/TTU Health Sciences Center
Box 41163
Lubbock, TX 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: 2/07/03

1. **DESCRIPTIVE STUDY TITLE:**
Perchlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

2. **STUDY NUMBER:** AQUA-03-01

3. **SPONSOR:**

United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

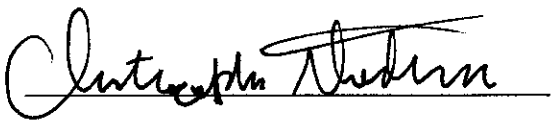
4. **TESTING FACILITY NAME & ADDRESS:**

Texas Tech University
The Institute of Environmental and Human Health
Box 41163
Lubbock TX 79406-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: 2/07/03
Termination Date: 12/31/03

6. **KEY PERSONNEL:**
Dr. Christopher Theodorakis, Study Director
Dr. Todd Anderson, Analytical Chemist
Dr. Ronald Kendall, Testing Facilities Management/Principal Investigator
Ryan Bounds, Quality Assurance Manager
Fujun Liu, Technician
Leslie McDaniel, Technician
June-Woo Park, Technician

7. **DATED SIGNATURES:**



4/2/03

Dr. Christopher Theodorakis
Study Director

Ronald Kendall	4-11-03	Dr. Ronald Kendall Testing Facility Management/PI
	4-2-03	Ryan Bounds Quality Assurance Manager
Todd Anderson	4-4-03	Dr. Todd Anderson Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to TIEHH and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:
Dr. Ronald J. Kendall
Texas Tech University
The Institute of Environmental and Human Health
Lubbock, TX 79409-1163 USA

9. STUDY OBJECTIVES / PURPOSE:

-To determine accumulation of perchlorate in invertebrates, detritus, and periphyton from the Texas Naval Weapons Industrial Reserve Plant (NWIRP).
-To determine concentrations of perchlorate in stomach contents of fish collected from NWIRP.

10. TEST MATERIALS:

Test Chemical name: Perchlorate anion
CAS number: 7790-98-9
Characterization: Determination of concentration in environmental samples.
Source: Wastewater treatment effluent discharge.

11. JUSTIFICATION OF TEST SYSTEM

Preliminary surveys of NWIRP have revealed that measurable levels of ammonium perchlorate have been found in surface waters of aquatic systems (i.e., streams, lakes, and

rivers) within and adjacent to NWIRP. Comparative analysis of perchlorate accumulation in fish and water from field-collected specimens indicates that perchlorate levels may be higher in fish than in water, but laboratory analysis indicates that perchlorate does not bioconcentrate in fish. This suggests that fish in the field are being exposed by some route other than direct absorption from the water. Possibly fish are being exposed through the food chain. In the stream ecosystem that have been studied so far, there is little phytoplankton, unlike lakes and larger rivers. Thus the primary productivity ("bottom of the food chain") comes from periphyton (algae film growing on rocks and other solid objects) or introduction of detritus (dead and decaying leaf litter) from terrestrial sources, and the associated saprobes (decomposers: bacteria and fungi) that grow on the detritus. Thus, one possible route of exposure of the fish is via accumulation of perchlorate by the periphyton or saprobes. Also, previous research has found that perchlorate accumulates in terrestrial plants, and most detritus in stream systems comes from fallen leaves of terrestrial plants in the riparian zone (i.e. streamside).
In addition, many fish in stream habitats, especially the ones that have been analyzed so far, feed on aquatic invertebrates. Uptake and possible accumulation from the water has not yet been addressed in invertebrates. Many invertebrates also feed upon detritus, and so could accumulate perchlorate from the detritus, which could then be passed on to fish, but the degree to which invertebrates accumulate perchlorate is currently unknown.
The uptake and bioaccumulation of perchlorate in invertebrates, periphyton, and detritus at perchlorate-contaminated sites has not been studied to date. There is also little information related to the possible uptake of perchlorate via ingestion of contaminated food. Therefore, the purpose of this study is to examine the levels of perchlorate in periphyton, detritus, invertebrates, and stomach contents in fish from NWIRP

12. TEST ORGANISMS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system):

Species: Carp (*Cyprinus carpio*) , sunfish and bass (*Lepomis spp., Pomoxis spp, Micropterus spp.*) catfish (*Ictalurus spp., Amerius ssp.*), or any other fish species deemed suitable as determined by abundance of specific aspects of their biology (to be determined on-site); periphyton and invertebrates (species dependant upon what is present and collected on site).

Strain: Wild animals.

Age: Various

Number: Maximum of 300 per species, taxon (invertebrates) or matrix (detritus).

Source: Captures from natural waters at NWRP.

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system will consist of natural waters within NWIRP. TTU has identified contaminated sites in previous surveys. Reference sites were selected based on proximity to similarity to NWIRP water bodies, and were not found to contain detectible levels of perchlorate. Each sampling location will be labeled with its whole name or a 4-letter abbreviation. To date, six sites have been identified. Their names (and 4 letter abbreviations) are Harris Creek (HARC), North Fork South Bosque River (NFSB), South Fork South Bosque River (SFSB), Wasp Creek (WASP), Coryell Creek (CORC), and Station Creek (STAC). Any other sites that may be added will be referenced either by their full name of the 4 letter abbreviation, determined as follow: the names of ponds, creeks, etc, will be abbreviated with the 1st three letters of the name followed by P (pond), C (creek), R (river), L (lake), or B (bayou) (e.g., Jim's Bayou = JIMB, Caddo Lake = CADL). If the name of the creek, lake, etc has only 4 letters, this may be used in place of a 4-letter abbreviation (Star Pond = STAR). If the water body consists of 2 or more words, the last letter of the abbreviation will indicate type of water body (P=pond, etc.), and the other letters will at least represent the first letter of each word of a compound name, and additional letters in the name may be added to total 4 letters, if needed (e.g., Little Cypress Bayou may be abbreviated LCYB or LICB, provided the same abbreviation is used for all samples; East Fork Poplar Creek would be abbreviated EFPC). If the name of the water body contains more than 4 words, the abbreviation of the last word may be omitted (e.g., for North Fork South Bosque River the "R" for river is omitted), All samples taken from the same water body within 100 meters may be counted as a single sample. If 2 or more samples are taken at intervals greater than 100 meters, or if a series of samples are taken from a stretch of lake, creek, etc, that is more than 100 meters long; the samples will be suffixed with numbers (e.g., HAGC-1, HAGC-2, etc.). If a pond, lake, creek does not have a name associated with it, it will be labeled with a letter, e.g., Pond A, Pond B, Lake A, Creek A, Creek B, Creek C, etc. The 1st 3 letters of the abbreviation will be PND (pond), CRK (creek), LKE (Lake), BYU (bayou) or RIV (river), followed by A, B, C, etc. (e.g., Pond A = PNDA). All names and abbreviations must be recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fish will be collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as can be collected will be taken from each site, up to 20 individual fish, large invertebrates, or composite samples per site [NOTE: herein, composite samples of detritus, invertebrates, or periphyton will be referred to as "composite samples"]. Individuals will be temporarily held in a bucket until processed.

Different buckets will be used for each site, if possible, or else buckets will be washed between sampling sites. Reference sites will be chosen so as to be as similar as possible to the contaminated site(s) in terms of habitat structure and stream characteristics. The same species, taxa, or matrices will be collected from contaminated and reference sites. Fish and composite samples will be weighed prior to processing and weight will be recorded on sampling form.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Water Sampling

At each location where fish are captured, 60 ml of water will also be taken for perchlorate analysis, according to SOP AQ-3-03. Water should be collected before collection of fish or composite samples, if at all possible. Water samples should be collected at either end of the section of the stream from which fish were collected, plus at least one sample in between these two points. Samples should be taken at least 10 m apart, unless the section of stream to be sampled is less than 10 m long. Water samples will be collected in precleaned glass vials (Wheaton), and will be collected from just under the water surface. Water samples should be stored away from direct sunlight and excessive heat (> 50° C). Before samples are taken, the pH, dissolved oxygen, conductivity, and temperature should be measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

Fish Collection

Fish may be collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps; or they may be collected with a seine, dip net or baited traps as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Traps should be placed at least 1 m. apart and checked at most every 24 hours. Traps should be anchored to a non-movable object on the shore with highly visible nylon twine or firmly attached to a highly visible floating buoy and anchored to the bottom. Placement of traps may be regularly spaced, or concentrated in habitats where target species are known to occur. If the water is too deep for backpack shocking, shocking may be done by boat. In smaller water bodies, the backpack shocker generator and power supply may be disconnected from the backpack frame and secured on the boat. In larger bodies of water, a boat electroshocking device may be used. Any captured fish will be placed in plastic buckets with aeration until processing.

Periphyton/detritus collection

Samples of detritus and periphyton will be collected by grab sampling. At least three samples will be collected from each water body sampled. Each sample will weigh at least

5 g and each sample will be collected at least 5 m apart. Samples will be placed in plastic bags or wrapped in aluminum foil, labeled according to SOP IN-3-02, and will be kept on ice or frozen in liquid nitrogen until transport back to the laboratory.

Invertebrate collection
Aquatic, benthic, or emergent insects will be captured with kick nets, seines, dip nets, or minnow traps (depending upon habitat structure). Kicknets or seines will be used to sample aquatic vegetation, sand, or gravel substrates, if present. Dipnets will be used to sweep aquatic vegetation, if present. Dipnet/kicknet sweeps will cover an area of at least 1 m by 1 m, and there will be at least three sweeps per sampling site (if appropriate habitat is present), with each sweep being at least 5 m apart.

Minnow traps will be used to sample crayfish, using chicken livers or other animal-based baits. Traps will be set and checked as described for fish. Each minnow trap will be placed at least 10 m apart, with at least 3 minnow traps per sampling site.

If at least 50 g of detritus can collected, any invertebrates present will also be collected from the detritus. Grab samples of detritus will be collected from at least 3 locations per sampling site, with each location being at least 5 m apart. If the detritus has not been frozen, it will first be soaked in 70 % ethanol to kill any invertebrates, after which the detritus will be washed in plastic buckets to dislodge dead organisms. The detritus will be removed from the bucket, and the remaining water will be sieved through window screening to collect any dislodged invertebrates. All collected invertebrates will be placed in plastic bags or wrapped in aluminum foil and stored on wet ice or frozen in liquid nitrogen until further analysis.

15.2 Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.

Frequency: Perchlorate is discharged into surface waters in NWRP continuously from ground waters or runoff from rainfall events.

Route/Method of Application: Ingestion of absorbtion of perchlorate from water and natural food items.

Justification for Exposure Route: The organisms are exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples will be collected for determination of perchlorate concentrations wherever biota samples are collected.

15.3 Test System Observation

At every location from which water samples are taken, the following environmental parameters will be evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

15.4 Animal Sacrifice and Sample Collections

Data Recording and Sample Labeling

Prior to processing any samples, they will be given a unique ID number. Species and weight will be recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish length is also an optional parameter than can be recorded. Fish or sample weight will be measured on a portable balance to the nearest gram (if the fish weighs 10 grams or more) of the nearest 1/10 gram if it weighs less than 10 grams.

Prior to use, the scale needs to be calibrated according to IN-4-01, "Field Scale Operations and Maintenance" and calibration should be recorded on TIEHH Form 60, or in bound field notebook.

According to the SOP IN-3-02, the minimum information to be recorded on labels is the project number and unique ID. The unique ID will contain enough information to be able to identify the species and tissue. Date of collection, species, sex and tissue may also be included on the label. Pre-printed labels may be used, but if they are used on samples to be frozen or chilled on ice, the project number and unique ID must also be printed on the container with indelible ink.

The unique ID will follow the following format: The ID will be W-(4-letter abbreviation of the species [see SOP IN-1-06]) - (2-letter abbreviation of the sampling site) – (sample number). For example, W-YLBH-SB-1 is the unique ID for yellow bullhead #1 collected from the South Fork South Bosque River. For detritus, invertebrate, and periphyton composite samples, the 4-letter species abbreviation will be replaced by DETR, INVT, and PERI, respectively. Any crayfish or freshwater clams that are large enough to analyze individually will be designated by the 4 letter abbreviation CRAY and CLAM, respectively. The letter W will precede all samples ID, signifying "Waco", the closest major town to NWRP. The 2-letter abbreviation of the sampling site will be derived from the 1st and last letters of the 4-letter abbreviation. If this designation is already used, the

2nd and last letters of the 4-letter abbreviation shall be used. The abbreviations will be recorded in the field notebook and/or the data record forms. Sample numbers will be assigned in the order in which they are processed. If a sample is a composite, then the letter C will be added to the ID. For example: W-YLBH-SB-C-1 is a composite sample of yellow bullhead catfish collected from South Fork South Bosque River. If a sample is divided into subsamples, a suffix consisting of a decimal point and a number will be assigned. For example, if W-YLBH-SB-C-1 and is divided into 2 subsamples, the IDs for these subsamples would be W-YLBH-SB-C-1.1 and W-YLBH-SB-C-1.2. If a fish is dissected into constituent organs, the label of each sample will be suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
GI tract	GI
Stomach contents	SC

For example, W-YLBH-SB-1-HD would be head sample from fish W-YLBH-SB-1, and W-LMBA- SB -C -1.2-FL would be subsample #2 from the fillet of largemouth bass #1. Labels for whole bodies do not need to contain a suffix (e.g., W-YLBH-SB-1 implies this sample is a yellow bullhead whole body).

Perchlorate Analysis:

Largemouth bass, carp, catfish, and/or sunfish will be collected for perchlorate stomach content and body burden analysis. Fish collected will be anesthetized with an overdose of MS222 (0.5 g/L). The stomach contents and/or GI tract, as well as the head and/or at least a 5 g sample of the fillet will be collected and frozen in liquid nitrogen for perchlorate analysis. Alternatively, fish may be chilled on wet ice until transport back to the laboratory. Fish should not chilled on wet ice for more than 5 days before processing or being frozen. Individual fish will be wrapped in aluminum foil and labeled prior to freezing or chilling.

Composite samples of periphyton, detritus, and invertebrates (or individual crayfish) will be frozen in liquid nitrogen or stored on wet ice until transport to the laboratory.

After transport to the laboratory, samples will be stored in the freezer (temperature -20° C or colder) until analysis. The samples will then be analyzed for perchlorate according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate".

Water samples also are transported back to the laboratory for perchlorate analysis. Once in the laboratory, they will be stored in a refrigerator (4° C) until analysis. They will then be analyzed for perchlorate according to SOP AC-2-11 "Analysis of Perchlorate by IC".

15.5 Endpoint Analysis

Analysis of samples for perchlorate will be according to SOP AC-2-11 "Analysis of Perchlorate by IC".

16. PROPOSED STATISTICAL METHODS

All data will be checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances will be checked using (Bartlett's or Lavine's test. Comparisons between sites will be accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. Correlation coefficients will be used to determine if residue levels correlate with biomarkers, reproductive, and/or population data.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include information entered on Form No. 181 "Aquatic Sampling Form"; Form No. 182 "Fish Dissection Form" or Form No. 027 "Multiple Fish/Amphibian Dissection/Collection Form". Data on these forms will include identity, number, mass, sex and location of animals captured; and identity, amount and location or water or sediment samples collected. Alternatively, this information may be recorded in QA-approved, bound field notebooks. Additional records to be maintained include Form No. 026 "Aquatic Sample Tracking Log"; Form No. Scale calibration log; Form no. 64 b and 64c "Batched Sample tracking log"; any entries in laboratory and field notebooks; and raw data from perchlorate analysis, thyroid hormone analysis and thyroid histology.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Perchlorate concentrations in biota collected and water concentrations of perchlorate at sites from which these biota were collected.
Thyroid hormone concentrations in plasma and/or whole bodies of biota collected.
Description and enumeration of alterations of thyroid hormone structure as revealed by histological analysis.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before 31 March 2001. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request. All data, the protocol and a copy of the final report shall be archived at the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

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Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: _X___ Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: __X__ Protocol ____ SOP ____ Other _____
Title: Pechlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

Dated: 02-07-03
Document # (if appropriate): AQUA-03-01
Page #(s): 1-6, 9
Section #: Title
Text to reference: Pechlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

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Change in Document: Pechlorate in Invertebrates, Periphyton, and Detritus at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

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Justification and Impact on Study: Because of interfering ions and other compounds, perchlorate could not be quantified in fish tissue samples.

Section #: 9
Text to Reference: To determine accumulation of perchlorate in invertebrates, detritus, and periphyton from the Texas Naval Weapons Industrial Reserve Plant (NWIRP).

Change in Document: To determine comparative body burdens of perchlorate in detritus, periphyton, and invertebrates collected from surface waters at the Naval Weapons Industrial Reserve Plant (NWIRP).

Justification and Impact on Study: Because fish tissue samples could not be analyzed for perchlorate, resources were diverted to analysis of other samples.

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* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

Section#: 9

Text to Reference: To determine concentrations of perchlorate in stomach contents of fish collected from NWIRP

Change in Document: This portion of the study was deleted.

Justification and Impact on Study: Because of interfering ions and other compounds, perchlorate could not be quantified in fish tissue samples.

Section #: 12

Text to Reference: Carp (*Cyprinus carpio*) , sunfish and bass (*Lepomis spp., Pomoxis spp, Micropterus spp.*) catfish (*Ictalurus spp., Amerius ssp.*), or any other fish species deemed suitable as determined by abundance of specific aspects of their biology (to be determined on-site); periphyton and invertebrates (species dependant upon what is present and collected on site).

Change in Document: Detritus, periphyton and invertebrates (species dependant upon what is present and collected on site).

Justification and Impact on Study: Because of interfering ions and other compounds, perchlorate could not be quantified in fish tissue samples.

Section #: 14

Text to Reference: Fish will be collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as can be collected will be taken from each site, up to 20 individual fish, large invertebrates, or composite samples per site [NOTE: herein, composite samples of detritus, invertebrates, or periphyton will be referred to as "composite samples"]. Individuals will be temporarily held in a bucket until processed. Different buckets will be used for each site, if possible, or else buckets will be washed between sampling sites. Reference sites will be chosen so as to be as similar as possible to the contaminated site(s) in terms of habitat structure and stream characteristics. The same species, taxa, or matrices will be collected from contaminated and reference sites. Fish and composite samples will be weighed prior to processing and weight will be recorded on sampling form.

Change in Document: Samples were collected from sites of known perchlorate contamination. As many individuals as could be collected were taken from each site, up

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

to 30 individuals per species per site. Each composite sample was composed of specimens collected along an approximately 50 m stretch of stream, and these stretches were located at least 50 m apart from each other.

Justification and Impact on Study: Because of interfering ions and other compounds, perchlorate could not be quantified in fish tissue samples.

Section #: 15.1
Text to Reference: *Fish Collection*
Fish may be collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps; or they may be collected with a seine, dip net or baited traps as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Traps should be placed at least 1 m. apart and checked at most every 24 hours. Traps should be anchored to a non-movable object on the shore with highly visible nylon twine or firmly attached to a highly visible floating buoy and anchored to the bottom. Placement of traps may be regularly spaced, or concentrated in habitats where target species are known to occur. If the water is too deep for backpack shocking, shocking may be done by boat. In smaller water bodies, the backpack shocker generator and power supply may be disconnected from the backpack frame and secured on the boat. In larger bodies of water, a boat electroshocking device may be used. Any captured fish will be placed in plastic buckets with aeration until processing.

Change in Document: This section was deleted.

Justification and Impact on Study: Because of interfering ions and other compounds, perchlorate could not be quantified in fish tissue samples.

Submitted by: Signature: _____ Date: 3/23/04

Authorized by: Study Director: _____ Date: 3/23/04

Received by: Quality Assurance Unit: _____ Date: 3/24/04

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ___✓___ Amendment ___x___ Deviation ____ Addendums

(we) RB 1-18-04

Document Reference Information

Check One: _x___ Protocol ____ SOP ____ Other _____

Title: ____ Perchlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

Dated: _____

Document # (if appropriate): ___ AQUA 03-01 _____

Page #(s): ___7 8 (we) RB 1-18-04

Section #: ___15.4

Text to reference: _____

"The unique ID will follow the following format: The ID will be W-(4-letter abbreviation of the species [see SOP IN-1-06]) - (2-letter abbreviation of the sampling site) – (sample number). For example, W-YLBH-SB-1 is the unique ID for yellow bullhead #1 collected from the South Fork South Bosque River. For detritus, invertebrate, and periphyton composite samples, the 4-letter species abbreviation will be replaced by DETR, INVT, and PERI, respectively. Any crayfish or freshwater clams that are large enough to analyze individually will be designated by the 4 letter abbreviation CRAY and CLAM, respectively. The letter W will precede all samples ID, signifying "Waco", the closest major town to NWRP. The 2-letter abbreviation of the sampling site will be derived from the 1st and last letters of the 4-letter abbreviation. If this designation is already used, the 2nd and last letters of the 4-letter abbreviation shall be used. The abbreviations will be recorded in the field notebook and/or the data record forms. Sample numbers will be assigned in the order in which they are processed. If a sample is a composite, then the letter C will be added to the ID. For example: W-YLBH-SB-C-1 is a composite sample of yellow bullhead catfish collected from South Fork South Bosque River. If a sample is divided into subsamples, a suffix consisting of a decimal point and a number will be assigned. For example, if W-YLBH-SB-C-1 and is divided into 2 subsamples, the IDs for these subsamples would be W-YLBH-SB-C-1.1 and W-YLBH-SB-C-1.2. If a fish is dissected into constituent organs, the label of each sample will be suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
GI tract	GI

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

Stomach contents SC

For example, W-YLBH-SB-1-HD would be head sample from fish W-YLBH-SB-1, and W-LMBA- SB -C -1.2-FL would be subsample #2 from the fillet of largemouth bass #1. Labels for whole bodies do not need to contain a suffix (e.g., W-YLBH-SB-1 implies this sample is a yellow bullhead whole body).

Change in Document: _____

_____ The unique ID followed the following format: For samples collected from NWIRP, the ID was 4-letter abbreviation of the sampling site)-X-(sample [or composite] number), where X was "D" if the sample was detritus, "P" for periphyton, "C" for crayfish, "SN" for snail, "CL" for clam, or "I" for other invertebrate. If more than one species of invertebrate was collected, it was given an additional designation of A, B, C...eg. IA, IB, IC for "invertebrate A, invertebrate B, invertebrate C," etc.

Justification and Impact on Study: _____

____ This was deemed to be a more efficient method of labeling the samples. It made the study more efficient.

Submitted by: Signature: _____ Date: 3/15 (WE) CWT 3/18/04

Authorized by: Study Director: _____ Date: 3/18/04

Received by: Quality Assurance Unit: _____ Date: 3-18-04

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ✓ Amendment ~~x~~ Deviation ____ Addendums

(we) RB 3-18-04

Document Reference Information

Check One: _x___ Protocol ____ SOP ____ Other _____

Title: ____ Perchlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

Dated: _____

Document # (if appropriate): ___ AQUA 03-01 _____

Page #(s): ___7_____

Section #: ___15_____

Text to reference: _____

"If the detritus has not been frozen, it will first be soaked in 70 % ethanol to kill any invertebrates, after which the detritus will be washed in plastic buckets to dislodge dead organisms. The detritus will be removed from the bucket, and the remaining water will be sieved through window screening to collect any dislodged invertebrates."

Change in Document: _____

_____No invertebrates were collected from detritus

Justification and Impact on Study: _____

____ No invertebrates were observed on the detritus, so they were not collected. This had no impact on the study, as invertebrates were collected more efficiently by other means.

Submitted by: Signature: _____ Date: 3/8/04

Authorized by: Study Director: _____ Date: 3/8/04

Received by: Quality Assurance Unit: _____ Date: 3-18-04

* Sequentially numbered in order of the date that the change is effective

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Form No. 014 Rev. 3.06/00
Project No.: ___T9700.11_
*Change No: ____4_____

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ___✓___ Amendment ___~~*~~___ Deviation ____ Addendums

RB 3-18-04

Document Reference Information

Check One: _x___ Protocol ____ SOP ____ Other _____

Title: ____ Perchlorate in Invertebrates, Periphyton, Detritus, and Fish Stomach Contents at the Naval Weapons Industrial Reserve Plant, McLennan County, Texas

Dated: _____

Document # (if appropriate): ___ AQUA 03-01 _____

Page #(s): ___10_____

Section #: ___16_____

Text to reference: _____

"All data will be checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances will be checked using (Bartlett's or Lavine's test. Comparisons between sites will be accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. Correlation coefficients will be used to determine if residue levels correlate with biomarkers, reproductive, and/or population data."

Change in Document: _____

_____ No statistical analyses were performed..

Justification and Impact on Study: _____

____ This project was a survey, rather than hypothesis testing, so no statistical methods were implemented

---

Submitted by: Signature: _____ Date: 3/18/04

Authorized by: Study Director: _____ Date: 3/18/04

Received by: Quality Assurance Unit: _____ Date: 3/18/04

* Sequentially numbered in order of the date that the change is effective

29 MAR 2004

Evaluation of Pendrin Expression in the Offspring of Ammonium Perchlorate-Dosed Deer Mice

STUDY NUMBER:	DEMO-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	July 2, 2003
RESEARCH COMPLETION:	December 31, 2003

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Figure 1. Relative pendrin gene expression in deer mice kidney following exposure to ammonium perchlorate. **Page 10.**

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Ernest Smith, PhD — Date: 25 MAR 04

Angella Gentles — Date: 3/25/04

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 25, 2004
Date

1.0 DESCRIPTIVE STUDY TITLE:
Evaluation of Pendrin Expression in the offspring of Perchlorate-dosed Deer Mice

2.0 STUDY NUMBER: DEMO-03-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: July 2, 2003
Termination Date: December 31, 2003

6.0 KEY PERSONNEL:
Ernest Smith, CO-PI
Angella Gentles, CO-PI
Bharath Ramachandran, Study Director
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator and Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:
The objective of this study was to determine the effect of perchlorate on the expression of pendrin in the deer mouse kidney.

8.0 STUDY SUMMARY:
In this study deer mice pups were exposed to ammonium perchlorate at 3 concentrations: 0ppm, 58.9 ppm, and 117.9 ppm. The pups were euthanized at postnatal day 21 and the kidneys quickly removed and placed in liquid nitrogen. They were then processed to isolate total RNA, which was used in reverse transcription polymerase chain reaction (PCR) to generate the cDNA for pendrin. A parial deer mice specific cDNA sequence was obtained. The cDNA was used to generate deer mice specific probe and a set of reverse and forward primers thate were utilized in real time PCR to quantitate the relative pendrin gene expression in the kidney of treated and control animals. The results show that AP at concentrations of 117ppm and 58.9ppm, significantly ($P < 0.05$) reduced pnedrin relative gene expression in the kidney of the deer mouse. Exposure to AP at 117.9 ppm did not appear to decrease relative pendrin gene expression

compared to AP at the 58.9 ppm dose level. This represents the first indentification of pendrin in the deer mice and indicate that perchlorate and or the secondary effects of perchlorate on the thyroid hormone axis may have signifcant effects on other organ systems at the molecular level.

9.0 TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

10.0 JUSTIFICATION OF TEST SYSTEM

Deer mice were used in this project because they are ubiquitous, opportunistic and are sentinel for wildlife. They are also easily handled, permit large sample sizes, and are more economically feasible in a study of this nature.
Live animals are necessary because culture and computer models cannot simulate changes in general homeostasis. In addition, culture and computer models would not provide pertinent scientific data for future use in risk assessment.

11.0 TEST ANIMALS:

Species:	Deer Mice
Strain:	Wild type
Age:	Postnatal day (PND) 0-21)
Sex:	Males and Females
Number:	Deer mice = 23 pups and 18 pairs of adults
Source:	In house breeding colony

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage was labeled as indicated in TIEHH SOP IN-3-06; label included genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage was labeled to include sex of the individuals (if appropriate), date of birth of pups, date of exposure, the name of the test substance and its concentration. Rodents were ear marked with unique identification numbers according to SOP ET-3-18.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

This experiment consisted of 3 treatment groups: Control, 58.9ppm AP and117.9ppm AP. Each treatment group had 6 pairs of adult animals.

14.0 METHODS

14.1 Test System acquisition, quarantine, and acclimation.

Adult deer mice were obtained from our breeding colony at Texas Tech University. They were maintained in standard cages lined with sani-chip bedding and kept on a 16L: 8D light regimen.

14.2 Assignment of Animals to Study Group and Identification

Animals were arbitrarily assigned to 1 of 3 treatment groups upon selection from the colony.

14.3 Test Material Application

The adult mice were exposed to AP via drinking water. AP was dissolved in deionized water at the prescribe rate. The pups were exposed gestationally and lactationally.

Rates/concentrations:
AP was given at concentrations of 0ppm, 58.9ppm, and 117.9 ppm in water.
Frequency: AP-treated drinking water was provided *ad libitum* .

Route/Method of Application: The pups were exposed gestationally and lactationally.

14.4 Daily Observations

Animals were monitored daily for changes in general health. If any changes were observed, animals were treated according to the University Veterinarian.

14.5 Animal Euthanasia and Sample Collections

On PND 21, the pups were euthanized using carbon dioxide asphyxiation and selected tissues including the kidneys were quickly extirpated, wrapped in pre-labeled foil, and placed in liquid nitrogen.

RNA Isolation

Total RNA was extracted, according to manufacturer's procedure, from control and ammonium perchlorate-treated kidney using Trizol Reagent (BRL, Gaithersburg, MD). The tissues were homogenized in Trizol Reagent (0.5 mg tissue/ml Trizol) and incubated at room temperature (RT) for 5 min. Chloroform (0.2 ml/ml Trizol) was then added to each

tube. After this, the tubes were then shaken and incubated at RT for 3 min. They were then centrifuged at 12,000-x g for 15 min at 4 C. The aqueous phase was transferred to a new tube and 0.5 ml isopropyl alcohol (per ml supernatant) and incubated for 10 min at RT. These were then centrifuged at 12,000 x g for 10 min at 4 C. The supernatant was removed and the pellet washed with 75% ethanol. The pellets were air dried for 5-10 min and dissolved in 50µl nuclease-free water. The RNA concentration was then determined spectrophotometrically at 260nm.

cDNA Cloning, Sequencing and RT-PCR analysis of the deer mice mRNA

An aliquot of total mRNA (2µg) of Deer mouse kidney mRNA was reverse transcribed, using an oligodT-primed first-strand kit (Ambion, TX) to generate deer mice cDNA for amplification and gene isolation.

For PCR amplification, reverse and forward oligonucleotide primers were designed according to the sequence for pendrin in *Mus musculus*. PCR was carried out using a Failsafe kit (Epicentre, WI). PCR was conducted for 40 cycles of denaturation (92 C, 30 sec), annealing (53 C, 30 sec.) and extension (72 C, 45 sec.), with a 5 min final extension. The deer mouse-pendrin PCR product was sequenced for identification and verification using an ABI (Perkin Elmer) DNA sequencer by Texas Tech Biotechnolgy Center.

Subsequently, the deer mice specific cDNA sequence was submitted to ABI primer design software for the development of Taqman specific probes and primers for Real Time PCR quantification of mRNA equivalents (Smith et al., 2002).
Sense primer sequence – GTCCCCAAAGTGCCAATCC; anti-sense primer – ACTCCTACCACATCCAGGAAGGA;
Taqman probe – AGCCTGGTGCTGGACTGTGGAGCT.

Data was standardized against GAPDH and analyzed for statistical difference with ANOVA and a multi-comparisons test using MiniTab statistical software.

15.0 Results

mRNA isolated from deer mice kidney was successfully transcribed to cDNA. A partial cDNA sequence for pendrin was generated by PCR and using deer mice specific primers. The PCR product was sequence and compared to Genbank sequence. The deer mice specific sequence was determined to be 97% similarity to the cDNA sequence posted for human pendrin gene. Deer mice specific real time primer set and probe generated from the deer mice specific sequence were subsequently used successfully in real-time PCR to determine the effect of exposure AP on transcriptional expression of pendrin. The result is shown in Figure 1. Ammonium

perchlorate at 58.9 and 117.9 ppm, significantly suppressed transcriptional expression of pendrin in the kidneys relative to control animals. Ammonium perchlorate-treated kidneys had approximately one half the level of pendrin expressed in the control kidneys.

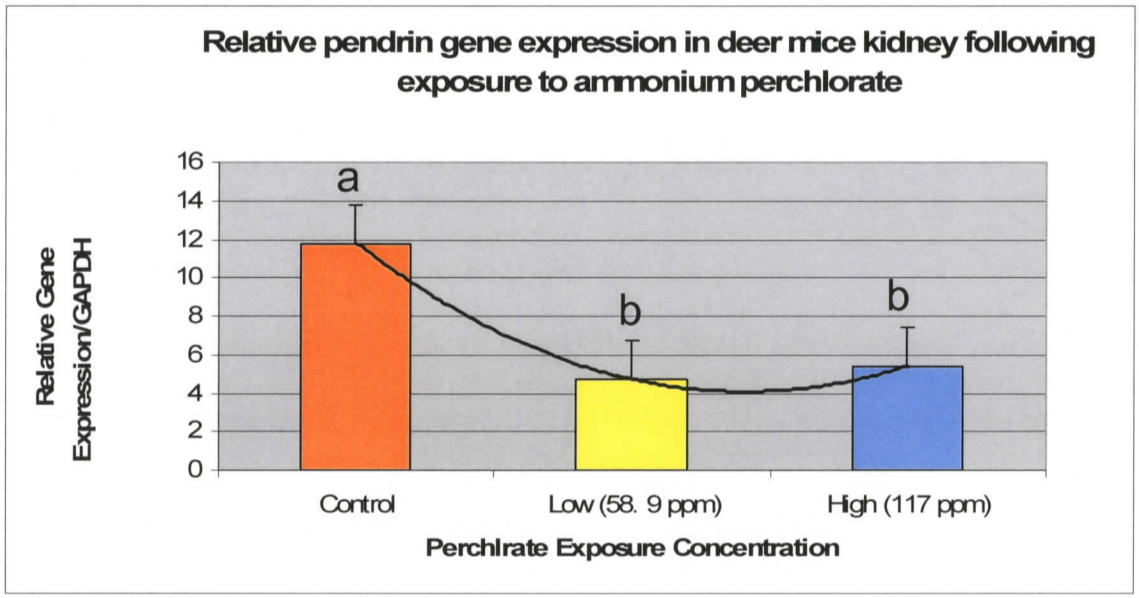

Figure 1

16.0 Discussion

The kidney plays a major role in maintaining and controlling systemic acid-base homeostasis by reabsorbing bicarbonate and secreting protons and acid-equivalents, respectively. During postnatal kidney development and adaptation to changing diets, plasma bicarbonate levels are increasing, the capacity for urinary acidification maturates, and the final morphology and distribution of intercalated cells is achieved (Bonnici and Wagner 2004). Variation in pendrin expression has been reported in the kidney during postnatal maturation. Pendrin is an anion exchanger expressed along the apical plasma membrane and apical cytoplasmic vesicles of type B and of non-A, non-B intercalated cells of the distal convoluted tubule, connecting tubule, cortical collecting duct, the thyroid and a few other tissues (Verlander et al., 2003; Frische et al., 2003; Wagner, et al., 2002; Wall et al., 2003). Pendrin is involved in iodide, chloride, and HCO(3)(-) secretion. Recently, Wall et al., (2003) reported that pendrin mRNA expression in the cortex is at least fivefold higher in CCD and the connecting tubule (CNT) than in the other segments and concluded that pendrin is expressed in the mouse distal convoluted tubule, CCD, and CNT along the apical plasma membrane of non-A-non-B intercalated cells and in subapical cytoplasmic vesicles of type B intercalated cells.

In this experiment, a partial sequence for pendrin was isolated from the kidney of the deer mouse. Submission of this cDNA sequence to GenBank revealed a 97% similarity to that of human. Real time PCR was used to determine the

relative expression of pendrin mRNA in the kidney of deer mice exposed to ammonium perchlorate. The result of this experiment shows that exposure to AP at 58.9 and 117.9 ppm, significantly suppressed transcriptional expression of pendrin in the kidneys. Ammonium perchlorate-treated kidneys had approximately one half the level of pendrin expressed in the control kidneys. Because pendrin transport HCO3- and Cl-anions, exposure to perchlorate could induce abnormalities in the acid-base and NaCl balance as well as blood pressure, and body weight. Furthermore, the altered regulation of pendrin suggests that metabolic alkalosis could be severely affected as has been demonstrated in pendrin-null transgenic mice (Verlander, et al., 2003) and could be critical in the pathogenesis of xenobiotic-induced hypertension. Further research in this area will help to elucidate the role of perchlorate and its effect on pendrin protein expression in the well-established regulation of HCO(3)(-) secretion in the CCD in response to chronic changes in acid-base balance and suggest that regulation of pendrin expression may be clinically important in the correction of acid-base disturbances (Frische, et al., 2003).

In the thyroid gland pendrin gene, localizes to the apical membrane of thyroid follicular cells and is thought to enable efflux of iodide into the follicle lumen, a process that genetic and/or environmental factors can influence in the thyroid as well as the kidney (Taylor et al., 2002). Ammonium perchlorate interference with the regulation of pendrin may also have pertinent implication in AP-thyrotoxicosis and the regulation of iodide metabolism. One contemporary mechanism for the toxicity of perchlorate anion, is that it competitively inhibits iodide transport via the sodium/iodide symporter in the thyroid follicular cells. The results from this study suggest that AP may inhibit thyroid hormone synthesis at the molecular level by suppressing pendrin; a gateway molecule involved in thyroid hormone synthesis.

Reference List

1. Bonnici, B. and Wagner, C. A. (2004). Postnatal expression of transport proteins involved in acid-base transport in mouse kidney. Pflugers Arch.

2. Everett, L. A., Morsli, H., Wu, D. K., and Green, E. D. (1999). Expression pattern of the mouse ortholog of the Pendred's syndrome gene (Pds) suggests a key role for pendrin in the inner ear. Proc Natl Acad Sci U S A **96**, 9727-32.

3. Frische, S., Kwon, T. H., Frokiaer, J., Madsen, K. M., and Nielsen, S. (2003a). Regulated expression of pendrin in rat kidney in response to chronic NH4Cl or NaHCO3 loading. Am J Physiol Renal Physiol **284**, F584-93.

4. Frische, S., Kwon, T. H., Frokiaer, J., Madsen, K. M., and Nielsen, S. (2003b). Regulated expression of pendrin in rat kidney in response to chronic NH4Cl or NaHCO3 loading. Am J Physiol Renal Physiol **284**, F584-93.

5. Lacroix, L., Mian, C., Caillou, B., Talbot, M., Filetti, S., Schlumberger, M., and Bidart, J. M. (2001). Na(+)/I(-) symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues. Eur J Endocrinol **144**, 297-302.

6. Scott, D. A., Wang, R., Kreman, T. M., Sheffield, V. C., and Karniski, L. P. (1999). The Pendred syndrome gene encodes a chloride-iodide transport protein. Nat Genet **21**, 440-3.

7. Taylor, J. P., Metcalfe, R. A., Watson, P. F., Weetman, A. P., and Trembath, R. C. (2002). Mutations of the PDS gene, encoding pendrin, are associated with protein mislocalization and loss of iodide efflux: implications for thyroid dysfunction in Pendred syndrome. J Clin Endocrinol Metab **87**, 1778-84.

8. Verlander, J. W., Hassell, K. A., Royaux, I. E., Glapion, D. M., Wang, M. E., Everett, L. A., Green, E. D., and Wall, S. M. (2003a). Deoxycorticosterone Upregulates PDS (Slc26a4) in Mouse Kidney. Role of Pendrin in Mineralocorticoid-Induced Hypertension. Hypertension.

9. Verlander, J. W., Hassell, K. A., Royaux, I. E., Glapion, D. M., Wang, M. E., Everett, L. A., Green, E. D., and Wall, S. M. (2003b). Deoxycorticosterone upregulates PDS (Slc26a4) in mouse kidney: role of pendrin in mineralocorticoid-induced hypertension. Hypertension **42**, 356-62.

10. Wagner, C. A., Finberg, K. E., Stehberger, P. A., Lifton, R. P., Giebisch, G. H., Aronson, P. S., and Geibel, J. P. (2002). Regulation of the expression of the Cl-/anion exchanger pendrin in mouse kidney by acid-base status. Kidney Int **62**, 2109-17.

11. Wall, S. M., Hassell, K. A., Royaux, I. E., Green, E. D., Chang, J. Y., Shipley, G. L., and Verlander, J. W. (2003). Localization of pendrin in mouse kidney. Am J Physiol Renal Physiol **284**, F229-41.

A STUDY PROTOCOL

ENTITLED

Evaluation Of Pendrin Expression In The Offspring Of Ammonium Perchlorate-Dosed Deer Mice

STUDY NUMBER: DEMO-03-01

SPONSOR: Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY
Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: *Bharath Ramachadran*

PROPOSED EXPERIMENTAL START DATE: JULY 2, 2003

1. **DESCRIPTIVE STUDY TITLE:**
Evaluation of Pendrin Expression in the offspring of Perchlorate-dosed Deer mice

2. **STUDY NUMBER:**
DEMO-03-01

3. **SPONSOR:**
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: July 2, 2003
Termination Date: December 31, 2003

6. **KEY PERSONNEL:**
Ernest E. Smith, Co-Principal Investigator
Angella Gentles, Co-PI
Bharath Ramachandran, Study Director
Lance Williams, Animal Care
Ryan Bounds, Quality Assurance Manager
James Surles, Statistical support
Ron Kendall, Principal Investigator and Testing Facility Management

7. **DATED SIGNATURES:**

_____	7/1/03	Dr. Ernest Smith Co-Principal Investigator
_____	7/1/03	Dr. Angella Gentles Co-PI
_____	7/1/03	Bharath Ramachandran, Study Director
_____	7-2-03	Mr. Ryan Bounds Quality Assurance Manager

7/7/03 Dr. James Surles
Statistical Support

7-2-03 Dr. Todd Anderson
Asst. Director - TIEHH

7-22-03 Dr. Ron Kendall
Testing Facility
Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in the spirit of Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

9. STUDY OBJECTIVES / PURPOSE:

To determine the effect of gestational and lactational exposure to ammonium perchlorate (AP) on pendrin expression in offspring of AP-treated deer mice.

10. STUDY SUMMARY:

In this study, adult deer mice will be exposed to AP and the effect of exposure on the expression of pendrin in their offspring will be analyzed. This experiment provides a realistic assessment of exposures that are likely to occur in environments contaminated with perchlorate.

11. TEST MATERIALS:

Test Chemical:	Ammonium Perchlorate 99.999% pure
Source:	Sigma-Aldrich
Characterization:	Oxidizer; explodes when heated
Test Medium:	Deionized Water

12. JUSTIFICATION OF TEST SYSTEM

This project is intended to evaluate risks of perchlorate exposure among organisms consuming perchlorate-contaminated water. Deer mice will be used as sentinels to wildlife and livestock (the most likely exposed organisms) because they permit large sample sizes and are economically and easily maintained.

This study represents the second phase of an ongoing project that requires live animals for each experiment and cannot be substituted with culture or computer generated models. Culture and computer models cannot simulate changes in general homeostasis and thyroid hormone alteration. In addition, they would not provide pertinent scientific data for furture use in risk assessment.

13. TEST ANIMALS:

Species: Deer mice

Strain:	Wild type
Age:	Weanlings
Sex:	Male and Female
Number:	Approximately 40
Source:	In house breeding colony

14. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each cage will be labeled as indicated in TIEHH SOP IN-3-06, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each cage will also be labeled to include sex of the individuals (if appropriate), date of birth, date of exposure, the name of the test substance and its concentration. Deer mice will be ear marked with unique identification numbers according to SOP ET-3-18.

15. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

This experiment will consist of 2 treatment groups; 0ppm AP (control) and 117ppm AP. Each treatment group will have 4 breeding pairs of animals.

16. METHODS:

Rodent Selection, Procurement and treatment group assignment: Sexually mature male and female deer mice will be selected from colonies housed in the AAALAC-accredited animal facility at Texas Tech University Human Sciences and randomly assigned to control or perchlorate-contaminated water. The effects of exposure to perchlorate will be evaluated at postnatal day 21 and 45 following gestational exposure.

Dosing rodents: Eight breeding pairs of deer mice will be arbitrarily assigned to control or perchlorate treatment group (ie 4 pairs per treatment). They will be housed in standard rodent cages (11 1/2 x 7 x 5 1/2) lined with aspen sani-chips. Exposure will be initiated the same day the animals will be paired. Water and food will be provided *ad libitum* throughout the experiment. Daily observations will be made on all deer mice. Animals showing signs of distress or disease will be treated according to the instructions of the University Veternarian.

Necropsy: At PNDs 21 and 45, pups will be narcosed by CO_2 and euthanization will be ensured by cervical dislocation. Blood samples will be collected by cardiac puncture. The plasma will be aliquoted and frozen (-80 °C) until hormone analysis. Gonads, thyroid and kidneys will be removed, trimmed and weighed. The collected tissue will be frozen for molecular analysis of pendrin mRNA levels. Liver samples will be removed and used for perchlorate exposure verification.

All deer mice handling will be done according to Standard Operating Procedures.

16.1 Test System acquisition, quarantine, and acclimation.
Adult deer mice will be obtained from our breeding colony at Texas Tech University. They will be maintained in standard cages lined with sani-chip bedding and kept on a 16L: 8D light regimen.

16.2 Assignment of Animals to Study Group and Identification
Animals will be assigned arbitrarily to treatment groups upon selection from the pool.

16.3 Test Material Application
Ammonium perchlorate will be dissolved in the drinking water of deer mice (SOP IN 3-05) at a concentration 117 ppm. Deer mice in the control group will be given untreated deionized water
Rates/concentrations: perchlorate (117 ppm) or no treatment.

Frequency: Test substances will be supplied *ad libitum* and renewed every 3 days.

Route/Method of Application: Adult deer mice will be exposed to AP orally via drinking water. The offsprings will be exposed to AP gestationally and lactationally.

16.4 Daily Observations
Animals will be monitored daily for changes in general health. Based on previous studies, no dead or moribund animals are expected to result from this exposure.

16.5 Evaluations
Selected organs (SOP ET-3-19) will be collected for evaluation of pendrin expression.

17. PROPOSED STATISTICAL METHODS

Organ weight and hormone concentration will be subjected to ANOVA and multi-comparison analysis.

18. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include animal receipt, animal care, test material preparation and application, animal observations, sex ratio results and facility records for personnel, equipment, etc.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Study Methods
Survival of treatment animals
Chemical analysis results
Interpretation of all data, including statistical results
Discussion of the relevance of findings

List of all SOPs used

19. RECORDS TO BE MAINTAINED LOCATION:

A final report containing the results of the dosing studies will be delivered to the Sponsor on or before March 20, 2004.. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility for up to 3 years.

20. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to affect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

21. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

22. REFERENCES:

Thuett et al., 2002. Journal of Environmental Toxicology and Health

TIEHH
Box 41163
Lubbock, TX 79409-1163
(806) 885-4567
qa@tiehh.ttu.edu

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment _X___Deviation ____ Addendums

Document Reference Information

Check One: _X___ Protocol ____ SOP ____ Other _____

Title: Evaluation of Pendrin Expression in the Offspring of Ammonium Perchlorate-Dosed Deer Mice
Dated: July 2, 2003
Document # (if appropriate):
Page #(s):
Section #:
Text to reference:

Change in Document:
It was indicated in the study protocol that 2 concentrations of AP would be used for this experiment. Three concentrations were used.

Justification and Impact on Study:
We need to determine if there was a dose-response relation; 2 levels of dosing could not provide such information.

Submitted by: Signature: Rsharath Rao Date: 03/23/04

Authorized by: Study Director: Rsharath Rao Date: 03/23/04

Received by: Quality Assurance Unit: Rick Hart Date: 03/24/04

A Final Report
Avian Exposure to Perchlorate – Field Study

STUDY NUMBER:	AFS-02-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Longhorn Army Ammunition Plant Karnack, Texas
RESEARCH INITIATION:	October 1, 2002
RESEARCH COMPLETION:	December 31, 2003

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Table 3. Summary for nesting activity of wood ducks in 2003 at the LHAAP, Karnack, Texas.

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of the Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Scott McMurry

Scott McMurry

3/25/04

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

3-26-04
Date

1.0 DESCRIPTIVE STUDY TITLE:

Avian exposure to perchlorate—field studies

2.0 STUDY/PROTOCOL NUMBER:

AFS-02-01

3.0 SPONSOR:

Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: October 1, 2002
Termination Date: December 31, 2003

6.0 KEY PERSONNEL:

Dr. Scott T. McMurry, Project Manager
Dr. Philip N. Smith, Project Manager
Mr. Ryan Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Principle Investigator / Testing Facility Management

7.0 STUDY OBJECTIVES /PURPOSE:

The data collected in 2003 was a continuation of the 2002 project designed to assess exposure to perchlorate in avian species. The focus of the study in 2003 was to obtain a more robust data set on perchlorate exposure in wood ducks (especially eggs) at the LHAAP. Objectives of the field studies in 2003 were to determine:

- The reproductive success of wood ducks (aquatic avian model) nesting at the LHAAP and survival and growth of their chicks.
- The level of perchlorate in wood duck eggs as an index of maternal transfer of perchlorate.

8.0 STUDY SUMMARY:

The Interagency Perchlorate Steering Committee (IPSC) identified avian exposure studies as a data gap in research efforts involving perchlorate. Given the lack of research on exposure and effects of perchlorate in avian species, several specific areas were identified as data gaps. These included assessing endpoints in birds such as levels of, and relationships between, perchlorate residues and hormone concentrations in adults. Also, the level of perchlorate transfer into eggs, reproductive success (e.g., number of clutches,

clutch size, egg viability, hatching rates), and recruitment of viable chicks (e.g., survival, growth, development) were all identified as data gaps. Other data gaps included evaluating food items as sources of exposure in birds, determining safe perchlorate levels in birds using controlled laboratory studies, and developing PBPK models for avian species. Our purpose is to aid in the filling of these data gaps.

In this study, we proposed to study avian exposure to perchlorate using aquatic and terrestrial avian species. Recent analyses of perchlorate residues in various plant and animal matrices indicates that significant exposure can occur from ingestion of aquatic invertebrates and aquatic and terrestrial plants (Smith et al, 2002). For example, composites of damselfly larvae averaged 1.5 ppm ($\pm$0.3, n=3 composite samples) perchlorate in contaminated impoundments at the LHAAP. Bulrush samples (n=4) from the same site averaged 7.6$\pm$1.4 and 4.4$\pm$2.2 ppm in above and below waterline samples, respectively. Sediment samples from this site averaged 25.1$\pm$6.8ppm perchlorate. Terrestrial plant samples (n=1 for each sample type) collected in 2002 near building 25C showed even higher concentrations of perchlorate, ranging from 6 ppm in stems of goldenrod to 5,557 ppm in blades of crabgrass. Seeds of these plants also contained significant amounts of perchlorate, with 1,880 ppm in crabgrass seeds and 184 ppm in goldenrod seeds. These data, although of limited sample size, indicate the potential for exposure through food chains by omnivorous wildlife. Based on the data presented above, we believe that avian species that consume both animal and plant material are at risk of exposure to perchlorate at contaminated sites. Avian species that use aquatic habitats are at risk of exposure to perchlorate through ingestion of aquatic invertebrates and aquatic plants. Likewise, terrestrial avian species are at similar risk of exposure.

9.0 TEST MATERIALS:

The test material is perchlorate in the environment.

10.0 JUSTIFICATION OF TEST SYSTEM:

Wood ducks (*Aix sponsa*) are year-round residents throughout the southeastern United States. They occur in abundance throughout Caddo Lake and its backwaters associated with the LHAAP, and are easily cultured in the field by erecting nest boxes in study areas. They consume a variety of plant and animal foods including the seeds, fruits, and vegetative material of aquatic and terrestrial plants (Landers et al., 1977; Drobney and Fredrickson, 1979; Delnicki and Reinecke, 1986). In addition, wood ducks consume a diverse number
of aquatic invertebrate species (Landers et al., 1977). Diet composition varies between males and females and breeding versus non-breeding females (Drobney and Fredrickson, 1979). In general, plant material comprises 50 to 60% of the diet for males and females, with the balance consisting of animal material. The major exception is egg-laying females that consume nearly 80% animal material.

Water and food consumption rates for wood ducks are unclear, but estimates can be derived from similar waterfowl such as mallards and lesser scaup, that consume about 6% of their body mass in water, and 8% (scaup) in food, each day. Given the variability in perchlorate concentrations in water, plant, and invertebrates at the LHAAP, clear

estimates of exposure are difficult to determine. However, water concentrations have been documented at 500ppb in Harrison Bayou-fed ponds. Concentrations of perchlorate for plant and animal samples were provided above. Based on these concentrations, a 600g wood duck could consume as much as 18µg of perchlorate per day from water consumption, and 46µg to 231mg of perchlorate per day from food consumption.

11.0 TEST ANIMALS:

Species: Wood duck

Strain: Wild

Age: Adult

Number: 20 wood duck eggs (collected in 2002 stored at -20°C and assayed in 2003), 8 wood duck eggs (collected and assayed in 2003).
1 merganser egg.

Source: Collected from perchlorate contaminated sites within the Longhorn Army Ammunition Plant, Texas.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Non-viable and early incubation wood duck eggs were collected and placed into uniquely identified bags including, but not limited to, date and nest box ID.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fields studies included monitoring of wood ducks on the LHAAP. Wood duck studies involved establishing artificial nesting structures through Harrison Bayou, extending from the INF pond toward Caddo Lake. Fifty nest boxes were initially established according to established methods (Bellrose and Holm, 1994). Spacing of the nest boxes was determined after initial site visits and identification of appropriate habitat. Wood ducks do not actively defend territories so they are amendable to nesting in high densities when provided artificial nest structures in clumped arrangements (Haramis, 1990). Exposure was monitored via perchlorate residues in eggs and diet items.

14.0 METHODS:

Sample Collection and Field Procedures

Longhorn Army Ammunition Plant (LHAAP), Texas

Wood duck nest boxes were erected at the LHAAP in late December 2001. Forty-nine boxes were initially established for the study, however in March 2003 the three boxes from the INF pond were removed from the study and one in Harrison Bayou was taken over by honey bees. Boxes distributed throughout Harrison Bayou, Goose Prairie Creek, Central Creek, and Star Ranch Pond were monitored in March and April of 2003. Nest box condition was monitored and recorded as to the presence of nesting material, eggs, adult birds, and chicks as well as other inhabitants.

Eggs were collected from all boxes with wood duck nesting activity. Since wood duck boxes were only monitored twice this year, the number of eggs collected was determined by the number of eggs in the box and nest box activity. All eggs were placed in plastic bags, labeled (nest box number, date) and frozen at –20°C until residue analysis was performed.

Sample analysis

In 2002, egg shells were opened and whole egg contents were analyzed without separating fetus from egg content. In 2003, egg shells were opened and the contents placed in a 1000mL beaker. If there was a developing fetus inside the egg, then it was analyzed separately from the rest of the egg contents. Eggs were analyzed for perchlorate content using standard tissue extraction and analysis techniques developed in the analytical core of this project (Anderson et al, 2002).

Statistical Methods

Data are typically presented as the mean and standard error. Detectable concentrations of perchlorate were scant and therefore we were unable to perform any significance tests.

15.0 RESULTS:

LHAAP, Texas: year 1 (2002)

Forty-nine wood duck nest boxes were monitored during the course of the breeding season in 2002 (Table 1). Of these, only six boxes showed any nesting activity by wood ducks. Other species seen in nest boxes included a hooded merganser, owls, and squirrels. However, these species were rare occurrences and most nest boxes had no activity. Of the six nest boxes with activity, one box was a single clutch, four boxes had double clutches, and one box had three clutches, for a total of twelve wood duck nests during the season. Mean clutch size was about fifteen eggs with an average of about four eggs hatched from each clutch, and a mean hatching rate of almost 36%. Virtually all of the nesting activity occurred in boxes in the Star Ranch Pond. Exceptions included a double clutch nest along Goose Prairie Creek, and a single clutch nest along Central Creek.

Table 1. Summary statistics for nesting activity of wood ducks in 2002 at the Longhorn Army Ammunition Plant, Karnack, Texas.

Total number of nest boxes	49
Nest boxes with wood duck activity	6
No. of boxes with single clutches	1
No. of boxes with double clutches	4
No. of boxes with triple clutches	1
Mean (+SE) clutch size	14.9±1.3
Mean (+SE) number of eggs hatched	4.3±1.4
Mean (+SE) percent hatch rate	35.6±10.5

A total of thirteen wood duck eggs, representing clutches from Star Ranch Pond, Central Creek, and Goose Prairie Creek, was analyzed for perchlorate in 2002. Two eggs, one each from the Central Creek and Goose Prairie Creek nests, had detectable concentrations of perchlorate, at 855 and 7,187 ppb (wet weight), respectively. All other eggs were non-detectable for perchlorate.

A total of twenty wood duck eggs, representing clutches from Star Ranch Pond, Central Creek, and Goose Prairie Creek that were collected in 2002 were analyzed for perchlorate in 2003. Five of the twenty analyzed were from the Central Creek and Goose Prairie Creek clutches that had detectable concentrations as reported in 2002. However, none of the twenty eggs collected in 2002 and assayed in 2003 had detectable concentrations of perchlorate.

Potential diet items of wood ducks were collected and analyzed for perchlorate (Table 2). In general, perchlorate was either not detected or found in trace amounts in most samples, including invertebrates, fish, tadpoles, crustaceans, and plants. Exceptions included 514 ppb perchlorate in fish from Harrison Bayou, and a range from 379 to 2,442 ppb in plants from a variety of sites including Harrison Bayou, Star Ranch Pond, and Central Creek. Logistical difficulties precluded collection of diet items from Goose Prairie Creek, but previous data indicate similar residue patterns from that area (Smith et al., 2001). These authors noted perchlorate concentrations approximately 100 ppb in fish from Goose Prairie Creek.

Table 2. Perchlorate concentrations in potential wood duck forage items collected from the LHAAP in Karnack, Texas in 2002.

		Concentration of perchlorate (ppb)					
Sample ID	Location	Invertebrates	Fish	Tadpoles	Crustaceans	Misc. plants	Aquatic plants
FA-1	SRP1	trace	trace	trace	--	ND	ND
FA-2	SRP	ND	ND	ND	ND	ND	trace
FA-3	HB 11/122	--	--	--	--	ND	--
FA-4	Boat ramp3	--	--	--	--	ND	--
FA-5	SRP	ND	trace	ND	--	--	trace
FA-6	CC4	ND	trace	trace	--	--	2442
FA-7	SRP	ND	trace	trace	--	ND	1292
FA-8	CC	trace	ND	trace	--	ND	722
FA-9	SRP creek5	ND	ND	--	Trace	ND	ND
FA-10	HB6	trace	trace	ND	Trace	trace	trace
FA-11	HB	ND	514	--	Trace	390	ND
FA-12	HB	--	--	--	--	trace	379

1SRP = Star Ranch Pond

2HB 11/12 = Harrison Bayou nest box numbers 11 and 12

3Boat ramp = Boat ramp on Caddo Lake northeast of SRP on the LHAAP.

4CC = Central creek

5SRP creek = tributary connecting SRP with Caddo Lake

6HB = Harrison Bayou

LHAAP, Texas: year 2 (2003)

Four nest boxes were eliminated from the study in 2003. Three nest boxes located at the INF pond were taken down due to no nest box activity and loss of water at the site. Box HB18 in Harrison Bayou is now inhabited by honey bees, and was not monitored for wood duck activity in 2003. Forty-five wood duck nest boxes were monitored during the course of the breeding season in 2003 (Table 3). Of these, only four boxes indicated any nesting activity by wood ducks. Other species seen in nest boxes included a hooded merganser (CC01), a screech owl (GP03), several flying squirrels (HB), and a couple of gray squirrels. Our March observations were as follows: CC01 was inhabited by a Merganser with twelve eggs and one was collected from the clutch; SRP3 had four warm eggs but no hen present at the time of observation; a total of five wood ducks were sighted during this two-day observation period. Our April monitoring was slightly inhibited by severe thunderstorms and lightning. April observations are as follows: SRP1 had fifteen eggs of which two eggs were collected, no hen was present but nest appeared to be tended; SRP2 had a wood duck incubating eighteen eggs of which two were collected; SRP3 became a dump nest with approximately forty eggs of which three were collected; SRP4 had one egg that was cold to the touch; a total of three wood ducks were sighted during this two-day observation period. All of the wood duck nesting activity occurred in nest boxes at Star Ranch Pond.

Table 3. Summary statistics for nesting activity of wood ducks in 2003 at the LHAAP, Karnack, Texas.

Total number of nest boxes	45
Nest boxes with wood duck activity	4

Eight eggs were collected from LHAAP during the spring of 2003. The one egg collected from the Merganser clutch and assayed in 2003 from the Central Creek nest (CC01) had a fetus inside of it. The egg contents had a detectable concentration of perchlorate, at 41 ppb (wet weight) but the fetus was non-detectable for perchlorate. All other eggs collected in 2003 were from wood ducks and were non-detectable for perchlorate.

16.0 DISCUSSION:

Birds represent a broad group of wildlife with a long history as sentinel species for assessing exposure to contaminants. The primary goal in the second year of this study was to obtain a more robust data set on wood ducks at the LHAAP. We expected this to be a feasible task given that the nest box array was in place throughout the preceding year, offering ample opportunity for wood ducks to

locate the boxes and establish nests. However, box occupancy rates were as equally low in the second year as the first year. The most likely explanation for low rates of box occupancy is high levels of available natural cavities throughout the LHAAP area and Caddo Lake site, which directly compete against artificial nest structure. Regardless of the cause, nest boxes in both years suffered from low occupancy rates despite being distributed throughout excellent wood duck habitat. The lack of occupancy had obvious ramifications on our ability to collect a range of samples from throughout the study site.

We attempted to alleviate the lack of available samples in 2003 by analyzing additional samples from 2002, as well as egg samples from 2003. The majority of our egg samples collected in either year did not show any detectable levels of perchlorate. Only two wood duck eggs and one merganser egg had any perchlorate. It is not completely surprising that only a few eggs (or one in these cases) would have measurable contaminant concentrations out of the entire clutch. Similar observations have been observed in other oviparous species (Wu, 2000). This observation in contaminant variation among eggs is probably linked to the temporal dynamics of exposure in the female coupled with the temporal process of egg development (i.e. exposure in the hen occurring at the proper time for incorporation of the contaminant in the egg). Nonetheless, perchlorate is capable of moving from the hen into her eggs. A similar study on a site with more widespread and higher levels of perchlorate would likely result in a greater number of eggs with detectable levels of the contaminant. During much of the 2002 and 2003 field seasons, perchlorate was being actively removed from the LHAAP site, effectly eliminating much of the potential exposure. As reported in our 2002 report, birds inhabiting other perchlorate-contaminated sites where remediation is not occurring readily showed detectable concentrations of perchlorate in their tissues.

Overall, a variety of different species of birds are capable of accumulating perchlorate from the environment. The effects of these exposures are unclear at this time. Comparison of the residue levels observed in this study with controlled laboratory studies will be instrumental in estimating the potential risk of perchlorate exposure to wild species of birds.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Anderson TA, Wu TH. 2002. Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. *Bull Environ Contam and Toxicol.* 68:684-691.

Bellrose FC and Holm DJ. 1994. Ecology and management of the Wood Duck. Stackpole Books, Harrisburg, PA.

Delnicki D Jr. and Reinecke KJ. 1986. Mid-winter food use and body weights of Mallards and Wood Ducks in Mississippi. J. Wildl. Manage. 50: 43-51.

Drobney RD and Fredrickson LH. 1979. Food selection by Wood Ducks in relation to breeding status. J. Wildl. Manage. 43: 109-120.

Haramis GM. 1990. Breeding ecology of the Wood Duck: a review. Pp 45-60 in Proc. 1988 N. Am. Wood Duck symp. St. Louis, MO.

Landers JL, Fendley TT, and Johnson AS. 1977. Feeding ecology of Wood Ducks in South Carolina. J. Wildl. Manage. 53: 378-382.

Smith PN, Theodorakis CW, Anderson TA, and Kendall RJ. 2001. Preliminary Assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. *Ecotoxicology.* 10: 305-313.

Tian K, Dasgupta PK, and Anderson TA. 2003. Determination of trace Perchlorate in high-salinity water samples by ion chromatography with line preconcentration and preelution. *Analytical Chemistry.* 75: 701-706.

Wu, T. 2000. Evaluation of organochlorine residues in Morelet's and American crocodile eggs from Belize. M.S. thesis, Texas Tech University, Lubbock, Texas. 120pp.

Project No. T9700

A STUDY PROTOCOL

ENTITLED

Avian Exposure to Perchlorate—Field Studies

STUDY/PROTOCOL NUMBER: AFS-02-01

SPONSOR:

Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

TESTING FACILITY:

Name/Address:

The Institute of Environmental & Human Health (TIEHH)
Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

Test Facility Management:

Dr. Ronald J. Kendall
Director, TIEHH

Study Director:

Dr. Scott T. McMurry
Dr. Phil N. Smith

PROPOSED EXPERIMENTAL
START DATE: JANUARY 1, 2002

1. **DESCRIPTIVE STUDY TITLE:**
Avian exposure to perchlorate—field studies

2. **STUDY NUMBER: AFS-02-01**

3. **SPONSOR:**
Strategic Environmental and Research
Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental & Human Health (TIEHH)
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: January 1, 2002
Termination Date: ~~September 30, 2002~~ December 31, 2003 (WE) RMS

6. **KEY PERSONNEL:**
Dr. Scott T. McMurry, Study Director
Dr. Philip N. Smith, Co-Investigator
Dr. Todd Anderson, Analytical Chemist,
Mr. Ryan M. Bounds, Quality Assurance Manager
Dr. Ronald J. Kendall, Primary Investigator / Testing Facility Management

7. **DATED SIGNATURES:**

Scott McMurry _____ Dr. Scott T. McMurry
Study Director

Ron Kendall _____ Dr. Ron Kendall
Testing Facility Management

_____ _____ Mr. Ryan Bounds
Quality Assurance Manager

Todd Anderson _____ Dr. Todd Anderson
Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to TIEHH and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. Scott T. McMurry
TIEHH
Box 41163
Lubbock, Texas 79409-1163

9. STUDY OBJECTIVES / PURPOSE:

The focus of this study is to obtain a more robust data set on perchlorate exposure in wood ducks (especially eggs) at the LHAAP. Objectives of the field studies are to determine:

- The reproductive success of wood ducks (aquatic avian model) nesting at the LHAAP and survival and growth of their chicks.
- The level of perchlorate in wood duck eggs as an index of maternal transfer of perchlorate.

10. JUSTIFICATION OF TEST SYSTEM

Wood ducks (*Aix sponsa*) are year-round residents throughout the southeastern United States. They occur in abundance throughout Caddo Lake and its backwaters associated with the LHAAP, and are easily cultured in the field by erecting nest boxes in study areas. They consume a variety of plant and animal foods including the seeds, fruits, and vegetative material of aquatic and terrestrial plants (Landers et al., 1977; Drobney and Fredrickson, 1979; Delnicki and Reinecke, 1986). In addition, wood ducks consume a diverse number of aquatic invertebrate species (Landers et al., 1977). Diet composition varies between males and females and breeding versus non-breeding females (Drobney

and Fredrickson, 1979). In general, plant material comprises 50 to 60% of the diet for males and females, with the balance consisting of animal material. The major exception is egg-laying females that consume nearly 80% animal material.

Water and food consumption rates for wood ducks are unclear, but estimates can be derived from similar waterfowl such as mallards and lesser scaup, that consume about 6% of their body mass in water, and 8% (scaup) in food, each day. Given the variability in perchlorate concentrations in water, plant, and invertebrates at the LHAAP, clear estimates of exposure are difficult to determine. However, water concentrations have been documented at 500ppb in Harrison Bayou-fed ponds. Concentrations of perchlorate for plant and animal samples were provided above. Based on these concentrations, a 600g wood duck could consume as much as 18µg of perchlorate per day from water consumption, and 46µg to 231mg of perchlorate per day from food consumption.

11. TEST ANIMALS:

Species: Wood duck

Strain: Wild

Age: Eggs

Number: Approximately 50 wood duck eggs

Source: Collected from perchlorate contaminated sites within the Longhorn Army Ammunition Plant, Texas.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Non-viable and early incubation wood duck eggs will be collected and placed into uniquely identified bags including, but not limited to, date and nest box ID.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fields studies include monitoring of wood ducks on the LHAAP. Wood duck studies involve establishing artificial nesting structures through Harrison Bayou, extending from the INF pond toward Caddo Lake. Fifty nest boxes will be initially established according to established methods (Bellrose and Holm, 1994). Spacing of the nest boxes will be determined after initial site visits and identification of appropriate habitat. Wood ducks do not actively defend territories so they are amendable to nesting in high densities when

provided artificial nest structures in clumped arrangements (Haramis, 1990). Exposure will be monitored via perchlorate residues in eggs and diet items.

14. METHODS:

Sample Collection and Field Procedures

Longhorn Army Ammunition Plant (LHAAP), Texas

Wood duck nest boxes will be erected at the LHAAP. Forty-nine boxes will be initially established for the study. Nest box condition will be monitored and recorded as to the presence of nesting material, eggs, adult birds, and chicks as well as other inhabitants.

Non-viable eggs will be collected from all boxes with wood duck nesting activity. All eggs will be placed in plastic bags, labeled (nest box number, date) and frozen at –20°C until residue analysis can be performed.

Sample Analysis

Residue analysis will be attempted on eggs, but given the high level of organic constituents in eggs, extraction procedures are expected to be difficult. Currently, we are using ion exchange methods to clean biologic samples, and significant amounts of organics (as found in eggs) may foul ion membranes. We will attempt to analyze perchlorate by extracting homogenates of yolk, albumin, and yolk and albumin combined.

15. PROPOSED STATISTICAL METHODS

Analysis of variance techniques may be used to evaluate differences in embryonic development among areas considered contaminated and those designated as clean.

16. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include field capture data, sample collection and handling logs, GPS coordinates of all collections, analytical data, embryonic growth and development.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- Collection success
- Serum perchlorate concentrations

- Developmental anomalies
- Embryonic growth and development

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

17. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before March 31, 2004. Copies of all data, documentation, records, protocol information, and the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

18. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

19. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

20. REFERENCES:

Delnicki and Reinecke, 1986
Drobney and Fredrickson, 1979
Hepp and Belrose, 1995
Landers et al., 1977
Haramis, 1990
Smith, 2000

29 MAR 2004

ANALYTICAL EVALUATIONS IN SUPPORT OF TOXICOLOGICAL INVESTIGATIONS

STUDY NUMBER:	ANALYT-03-01
SPONSOR:	Strategic Environmental Research and Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	9/1/2002
RESEARCH COMPLETION:	12/31/2003

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Figure 1. Chromatograms obtained by PC/PE and EPA Method 314.0, 1.0 mL samples

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Todd Anderson
Todd A. Anderson, Ph.D.

3-26-04
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 25, 2004
Date

1.0 DESCRIPTIVE STUDY TITLE:
Analytical Evaluations in Support of Toxicological Investigations

2.0 STUDY NUMBER:
ANALYT-03-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303

4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start: 9/1/02
Termination: 12/31/03

6.0 KEY PERSONNEL:
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
The focus of the subproject outlined here was to provide analytical support for ongoing toxicological evaluations of perchlorate in fish, amphibians, and mammals. In addition, because of the paucity of relevant food chain data on perchlorate, basic environmental analyses of potential food items (vegetation and seeds) were also carried out. Our goal was to support laboratory and field studies by measuring and quantifying potential perchlorate exposure among organisms.

8.0 STUDY SUMMARY:
Perchlorate concentrations in various samples were determined using ion chromatography. Samples submitted for analysis included soil, sediment, water, plant and vegetable matter, seeds, and various biological tissues and fluids. These analyses utilized equipment and analytical methods developed and validated during previous studies on perchlorate carried out by this laboratory.

A variety of tests were used throughout the course of sample analysis to ensure optimum performance of the analytical instrument as well as the data generated. These tests

included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others.

9.0 TEST MATERIALS:

Test Material: Laboratory and Environmental Samples

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: NIST-Certified.
Source: AccuStandard, Inc.

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: ACS-Certified.
Source: Fisher Scientific, Inc.

Test Chemical: Ammonium Perchlorate
CAS Number : 7790-98-9
Characterization: ACS-Certified.
Source: Aldrich, Inc.

Reference Chemical: deionized water (18MΩ)
CAS Number : NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

Evaluating tissue levels and identifying possible sources of perchlorate contamination are critical in determining toxicological and ecological exposure. The unique characteristics of perchlorate make accurate quantitation in biological matrices difficult (Ellington and Evans, 2000). The presence of additional ions, proteins, lipids, and other biomolecules that can foul ion exchange columns further confounds accurate determination of perchlorate concentrations in biological tissues and fluids.

11.0 TEST ANIMALS:

NA.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Samples were logged in at the time of receipt and logged out at the time of analysis. As the samples were not provided in any particular order (chronologically), they were not analyzed in any particular order. Sample tracking numbers and dates were included in log sheets.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

A variety of tests were used throughout the course of sample analysis to ensure optimum performance of the analytical instrument as well as the quality of the data generated

(Skoog and Leary, 1992). These tests included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others.

14.0 METHODS:

Dionex Corp. (1998) originally developed an ion chromatography method which allowed detection of perchlorate in water down to 1 μg/L (ppb). We have used this method for analysis with modifications as necessary for extraction, cleanup, and detecting perchlorate in tissue and vegetation samples (Anderson and Wu, 2002; Tian et al., 2003). Ion chromatography is the technique most widely used for the determination of perchlorate because of its sensitivity, ability to separate perchlorate from other ions, and its availability in many laboratories. Nevertheless, trace determination of almost any analyte by IC in the presence of high concentrations of other anions can be difficult. The case of perchlorate is a little more favorable than the typical ionic analyte because perchlorate is more strongly retained than most anions. Still, high salinity samples cause a high signal background from tailing peaks of the less retained ions and lead to poor analyte recovery. In many cases, the matrix peak(s) will totally overlap the perchlorate response. In EPA Method 314.0, serial pretreatment by Ag^{+}-, Ba^{2+}-, and H^{+}-loaded cation exchangers are recommended for removal of chloride, sulfate, and carbonate. This approach is at best partially effective in our experience and is not easily automated. Moreover, this treatment is of little value with biological tissue and fluid samples. Earlier, we developed an online preconcentration/preelution (PC/PE) approach for improved IC analysis of the more difficult samples (Tian et al., 2003). A short hydrophilic column is used to preconcentrate perchlorate. A dilute NaOH solution is used to prewash the sample loaded in the preconcentrator column to remove the less strongly held anions prior to switching the preconcentration column to the main separation column. We have previously evaluated the operational conditions, linear dynamic range, LOD, applicable sample volume, and effects of sample conductivity, as well as comparing the performance of the system with EPA Method 314.0 (**Figure 1**).

General operation of the ion chromatograph (DX-500, Dionex Corp.) is described in **SOP AC-4-03**. The operation of the ion chromatograph for perchlorate analysis is described in **SOP AC-2-11** and **SOP AC-2-15**. These SOPs provided the basis for determining perchlorate in dosing solutions used in the toxicological tests. As described in **SOP AC-2-11**, the analysis of perchlorate using the Dionex instrument is controlled by PeakNet software using a method entered and saved within the software package.

15.0 RESULTS:

A variety of tests were used throughout the course of sample analysis to ensure optimum performance of the analytical instrument as well as the data generated (Skoog and Leary, 1992). These tests included calibration on the days of sample analysis, blank samples (DI Water), check standards, sample carryover analysis, and sample replicates among others. A summary of the individual data quality tests are described below.

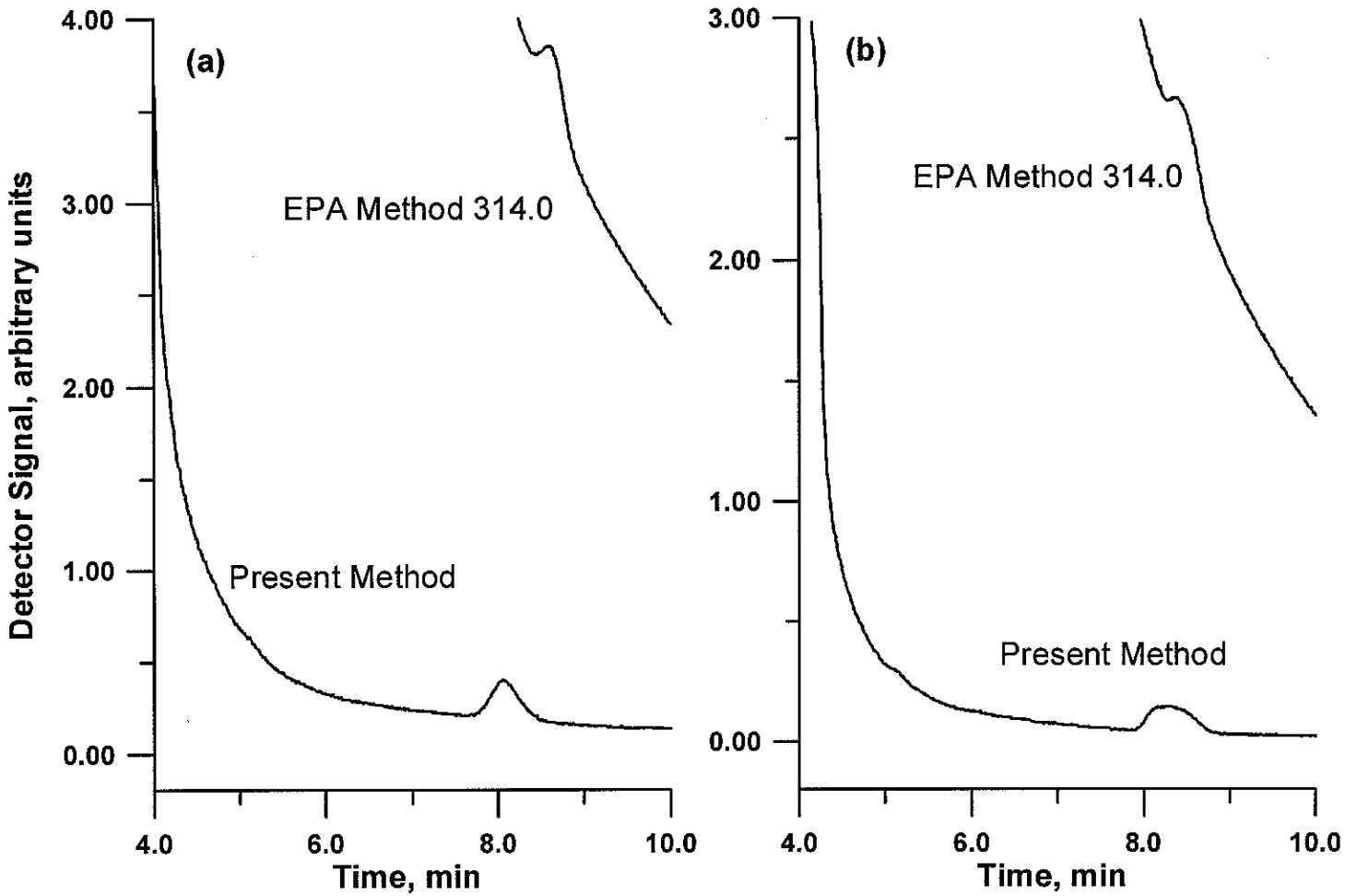

Figure 1. Chromatograms obtained by PC/PE and EPA Method 314.0, 1.0 mL samples (a) 25 µg/L perchlorate spiked into groundwater sample (κ = 4.7 mS/cm), present method prewash 2.3 mL 10 mM NaOH, (b) 25 µg/L perchlorate spiked into matrix containing 2000 mg/L each of Cl^-, SO_4^{2-} and CO_3^{2-} (κ = 14.7 mS/cm), present method prewash 2.7 mL 10 mM NaOH.

As a calibration curve is run each time a set of samples is analyzed, we routinely include an analysis of the calibration curves as part of our evaluation. We use a certified perchlorate standard (100 µg/mL) to determine possible dilution errors as well as to prepare calibration curve standards. These calibration curves represent the analysis of identical calibration standards on different days as well as different calibration standards (calibration standards expire after 60 days). Overall, variation in detector response for the calibration standards is low. As expected, the lowest calibration standard (2.5 ppb) has the highest %CV. This concentration is also our typical method limit of quantitation. The calibration curves are very linear over the range of calibration standards. The regression coefficient (r^2) for all of the calibration runs (> 2000) has never been below 0.995. This represents the r^2 from the untransformed data.

Throughout the analyses, we include check standards (calibration standards of known concentrations treated as samples) to ensure the performance of the calibration curve in calculating sample concentrations. In addition, we also perform individual check standard tests in which 2 perchlorate standards are analyzed repeatedly. This test also included DI water blanks in between each check standard. The results of these tests indicate low standard deviations and coefficient of variation. The values of SD and %CV for the check standards are typical for analytical measurements and represent the precision of the analytical method. The % differences between the actual and analytically determined concentrations are indicative of method accuracy expressed in terms of relative error.

As part of our analysis of sample reproducibility, we conduct tests using environmental samples. These tests also allow us to determine potential matrix effects for the aqueous samples. The %CV (precision) from these tests are always similar to those obtained in the check standard test described above.

As part of our analysis of sample reproducibility, we also conduct tests to determine possible dilution errors with calibration standards. For example, a 100 ppb perchlorate standard is diluted with DI water to make the following perchlorate sample array: 100 ppb, 50 ppb, 20 ppb, 10 ppb, 5 ppb, and 2.5 ppb. A perchlorate calibration curve is then used to determine the concentrations of the samples and these analytically determined concentrations are compared to the expected concentrations. The results of these tests indicate that analytically determined concentrations agree with expected concentrations indicating no dilution errors. The % differences (accuracy expressed in terms of relative error) from one test ranged from –6.56% to 2.43%. These values are typical and consistent with our check standard and replicate sample analyses.

16.0 DISCUSSION

More than 500 samples were analyzed for perchlorate in 2003. These samples included laboratory dosing solutions and exposure tank waters as well as environmental samples (water, vegetation, biological tissues). Samples were received from several research investigators (E. Smith, J. Carr, P. Smith, C. Theodorakis).

In summary, the perchlorate analyses we have conducted in support of toxicological investigations have been critically evaluated for precision and accuracy. These analyses were conducted utilizing equipment and analytical methods developed and validated during previous studies on perchlorate carried out by this laboratory.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Anderson, T. A., and T. H. Wu. 2002. Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. Bulletin of Environmental Contamination and Toxicology. 68:684-691.

Dionex. 1998. Application Note 121. Dionex Corp. Sunnyvale, CA.

Ellington, J. J., and J. J. Evans. 2000. Determination of perchlorate at parts-per-billion levels in plants by ion chromatography. Journal of Chromatography. 898:193-199.

Skoog, D. A., and J. J. Leary. 1992. Principles of Instrumental Analysis. 4th Ed. Harcourt Brace and Company. Orlando, FL.

Tian, K., P. K. Dasgupta, and T. A. Anderson. 2003. Simple determination of trace perchlorate in high salinity water samples by ion chromatography with online preconcentration and preelution. Analytical Chemistry. 75:701-706.

29 MAR 200

ENVIRONMENTAL MODELING

STUDY NUMBER:	MOD-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
ANIMAL TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	10/1/02
RESEARCH COMPLETION:	12/31/03

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Figure 4. Computer simulation for the transient temperature and vasomotor responses of a subject when exposed to a 16°C decrease in ambient temperature (28°C to 12°C) at 150 hrs (*pf*=0.60).

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Figure 8. Simulated thyroid tissue concentrations based on a 975 ppb dose of perchlorate with juvenile ingestion term

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Table 1. Thyroid hormone inhibition based on a 975 ppb dose of perchlorate starting at hour 1000

GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

Kenneth R. Dixon

3/25/04
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under The Institute of Environmental and Human Health Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 17, 1989.

Submitted By:

Quality Assurance Manager

March 25, 2004
Date

1.0 **DESCRIPTIVE STUDY TITLE:** ENVIRONMENTAL MODELING

2.0 STUDY NUMBER: MOD-03-01

3.0 SPONSOR: Strategic Environmental Research and Development Program
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4.0 TESTING FACILITY NAME AND ADDRESS:
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5.0 PROPOSED EXPERIMENT START AND TERMINATION DATES:

Start date:	10/01/02
Termination date:	12/31/03

6.0 KEY PERSONNEL
Kenneth R. Dixon
Eric P. Albers
Randy L. Apodaca
Srivatsa Patangi

7.0 STUDY OBJECTIVES/PURPOSE
Modeling and GIS will utilize the previously developed models to simulate the movement and effects of perchlorate at the LHAAP. A suite of models has been developed to simulate the transport, uptake, and effects of perchlorate in aquatic and terrestrial ecosystems. The emphasis in model development to date has been on uptake and distribution of perchlorate in mammal, fish, amphibian, bird, and plant species. Little information has been available on the effects of perchlorate on these species. The lab and field studies in this continuation will provide data that can enhance the effects aspects of these models.

We also will complete the implementation of the suite of models to provide for large-scale simulations, including estimates of risk for a risk assessment of the LHAAP site. This integrated suite of models will be available to assess perchlorate effects at other contaminated sites, and with modification, the effects of other contaminants.

Small Mammal Model. Basal metabolic and heart rate will be added as state variables in these models. Temperature will be added as a forcing function to predict the effects of perchlorate exposure on thermoregulation.

Fish Model. Food items for fish species and the perchlorate concentration in those items will be added to the fish model. The model will be calibrated using data collected at the LHAAP site.

Plant Model. Little is known about perchlorate transport mechanisms in plants. Lab and field studies on perchlorate exposure in plants will provide data to incorporate more mechanistic transport processes in the plant models. Measured perchlorate concentrations in different plant tissues will provide data for model calibration and validation.

8.0 METHODS

Small Mammal Model. To maintain body temperature, the physiological and metabolic reactions that produce heat must be balanced against those that radiate or conduct it away. Except within a range of ambient temperatures called the thermoneutral zone, maintaining a constant body temperature makes a steady demand either on the biochemical processes of heat production and/or the physical mechanisms for heat loss. When ambient temperatures fall below the thermoneutral zone (lower critical temperature or LCT), heat production must increase and/or heat loss must decrease. Above the other end of the thermoneutral zone (upper critical temperature or UCT), heat loss must increase (Ricklefs 1993).

The dynamics of the temperature regulation in mammals is a complex physiological process involving many factors. Ambient temperature is the predominant factor to trigger the regulation of metabolic rate and body temperature. Additionally, some drugs or environmental chemicals, such as perchlorate, also can affect the metabolic rate and body temperature in small mammals (Machle and Hatch. 1947). There have been several models to simulate temperature regulation in man exposed to various environmental conditions (e.g., Wyndham 1960 and Wissler 1961) have made some attempts by assuming the man to be a cylindrical physical model. We adapted a model by Crosbie (1961) who developed a model to simulate the regulation for cold responses in man. In this model, man was divided into three layers: surface (skin), middle (muscle), and core (rectal). Each layer was assumed to have a temperature which has a single value throughout its thickness. A separate ordinary differential equation was written for each layer in which time was the independent variable.

Each layer of Crosbie's model was considered to have uniform thermal properties, and the basal metabolic heat was considered to be generated in the inner-most layer. This latter is a necessary requirement since the core temperature, T_2, (all symbols and definition were listed in table 1) has a basal value higher than the more superficial temperatures in the body. Additional heat, caused by shivering or exercising, was considered to be generated in the middle layer that was comparable to the musculature of the body. The thickness of the layers was chosen to give a temperature difference

between core and skin sufficient to have a linear temperature gradient which would equal the basal metabolism. Heat flow was assumed to be unidirectional, and the core was assumed to be ideally insulated except from heat flow to the surface. Equations (2-4) give the amount of heat which was stored per unit time, per unit cross-sectional area, in the outer, middle, and inner layers, respectively.

$$\frac{dT_s}{dt} = \frac{K}{\rho c \Delta X_{s1} \Delta X_s}(T_1 - T_s) - \frac{0.1 * h}{\rho c \Delta X_s}(T_s - T_a) - \frac{0.1 * V}{\rho c \Delta X_s} \qquad (1)$$

$$\frac{dT_1}{dt} = \frac{K}{\rho c \Delta X_{12} \Delta X_1}(T_2 - T_1) - \frac{K}{\rho c \Delta X_{s1} \Delta X_s}(T_1 - T_s) - \frac{\Delta M}{\rho c \Delta X_1} \qquad (2)$$

$$\frac{dT_2}{dt} = \frac{M_0}{\rho c \Delta X_2} - \frac{300 * K}{\rho c \Delta X_{12} \Delta X_2}(T_2 - T_1) \qquad (3)$$

Note that the heat lost by radiation, convection, and vaporization are surface effects only and, therefore, occur only in Eq. (1). Also, the variation in the internal heat source ΔM, caused by shivering or exercising, originates in the middle or muscular layer and, therefore, occurs only in Eq.(2).

The average temperature of the body was calculated on a veighted basis, according to layer thickness as shown in Eq.(4).

$$T_B = \frac{\Delta X_s T_s + \Delta X_1 T_1 + \Delta X_2 T_2}{\Delta X_s + \Delta X_1 + \Delta X_2} \qquad (4)$$

To compare the physical model to the physiological situation that involves regulation, relations must be established between heat loss and body temperature. A study of steady-state data revealed that the thermal conductance of the peripheral tissues (which is related not only to specific tissue conductance but to blood flow and hence vasomotor activity) increases or decreases within limits as the body temperature rises or falls (Eqs. 5-6).

$$K = K_0\{1 + \alpha k + \Delta T_B + \gamma k \frac{dT_B}{dt}\} \leq 1.7K_0; \Delta T_B > 0 \qquad (5)$$

$$K = K_0\{1 + \alpha k - \Delta T_B + \gamma k \frac{dT_B}{dt}\} \geq 0.56K_0; \Delta T_B < 0 \qquad (6)$$

The steady-state data also show that the vaporization loss increases rapidly as the body temperature rises above its neutral value, but maintains a rather constant value as the body was cooled below this neutral temperature (Eqs. 7-8).

$$V = V_0 + \delta_E(\alpha_\upsilon \Delta T_B + \lambda_\upsilon \Delta T_B{}^4) \le 50V_0; \Delta T_B > 0 \qquad (7)$$

$$V = V_0; \Delta T_B < 0 \qquad (8)$$

The steady-state data further revealed that the body metabolism increases in proportion to the body temperature decrease when exposed to a cold environment. The body metabolism will also increase during exercise (Eqs. 9-10). Based on the effects of perchlorate on the metabolic rate, the metabolic rate coefficient of proportional control (α_m) was replaced by perchlorate factor (*pf*) in Eq. 11.

$$\Delta M = E; \Delta T_B > 0 \qquad (9)$$

$$\Delta M = -\alpha_m \Delta T_B + E; \Delta T_B < 0 \qquad (10)$$

$$\Delta M = -pf * \Delta T_B + E; \Delta T_B < 0 \qquad (11)$$

The surface conductance h was defined in Eq. 12 as being the sum of a radiation and a convection term. The convection term was further shown to vary as the square root of the surface air velocity. When $\upsilon = \upsilon_0$, $h = h_0$.

$$h = h_0 + \Delta h = h_r + h_c \sqrt{\frac{\upsilon}{\upsilon_0}} \qquad (12)$$

Table 1. List of symbols and Definitions.

ρ density of the tissue
c specific heat of tissue
ρc = 1 cal/cc°C
T temperature in degrees centigrade
Ts temperature of surface layer, Ts_0 = 33.6°C
T_1 temperature of middle layer, T_{10} = 34.7°C

T_2 temperature of core layer, T_{20} = 37.0°C
T_B average body temperature, T_{B0} = 35.8°C
T_α temperature of surface layer, $T_{\alpha 0}$ = 28 °C
t time
K specific thermal conductivity of wet tissue;
$K_0 = 1.1*10^{-3}$ cal/cm/°C = 0.4 kcal/m/hr/°C
αk^+ thermal conductivity coefficient of proportional control for $\Box T_B > 0$ = 0.147/°C
αk^- thermal conductivity coefficient of proportional control for $\Box T_B < 0$ = 0.066/°C
γk thermal conductivity coefficient of rate control in sec/°C ≃ 3.5 sec/°C
X distance in cm from the surface
ΔXs thickness of surface layer = 0.8 cm
ΔX_1 thickness of middle layer = 1.6 cm
ΔX_2 thickness of core layer = 3.2 cm
$\Delta Xs_1 = ½(\Box Xs + \Box X_1)$
$\Delta Xs_2 = ½(\Box X_1 + \Box X_2)$
M' metabolic rate per unit volume
M metabolic rate per unit area; M_0 = 37 kcal/m^2/hr
αM thermal conductivity coefficient of proportional control; $T_B < 0$
R' heat loss due to radiation effect per unit volume
R heat loss due to radiation effect per unit area
V' heat loss due to vaporization per unit volume
V heat loss due to vaporization per unit are; V_0 = 7 kcal/m^2/hr
αv vaporization coefficient of proportional control = 11 kcal/m^2/hr/°C; $T_B > 0$
λv vaporization coefficient of 4th power proportional control = 53 kcal/m^2/hr/°C^4; T_B>0
h heat transfer coefficient (skin to air) also known as surface conductance;
h_0 = kcal/m^2/hr/°C
h_r heat transfer coefficient of radiation = 0.48 h_0
h_c heat transfer coefficient of convection = 0.52 h_0
v velocity of air at skin surface; v_0 = 7.6 cm/sec
E exercise kcal/m^2/hr
δ_E increase in vaporization coefficient due to violent exercise

Fish Model. PBTK Model Description. A physiologically based toxicokinetic (PBTK) model was developed to simulate the movement of perchlorate within channel catfish. Contaminant movement was governed by a series of mass-balanced differential equations programmed in Matlab®. Model compartments and blood flow can be seen in Figure 1. General equations used in the model were taken from PBTKs developed for rainbow trout by Nichols et al. (1990, 1991) and channel catfish (Nichols, et al. 1993). Fish gill physiology was kept biologically accurate by accounting for countercurrent chemical flux, including both flow and diffusion limitations (Erickson and McKim 1990). Distribution was assumed to be flow-limited, i.e. chemical equilibrium existed between

the tissues and blood leaving the compartment. Additionally portal blood flow was incorporated into the kidney and liver from poorly perfused tissue and richly perfused tissue respectively. Portal blood flow to the kidney was set as 60% of blood flow to skin and muscle compartments, with portal flow to the liver equal to blood flow to the GI tract (Nichols et al. 1990). Finally an ingestion term was included to allow for the incorporation of multiple food sources of varying levels of toxicity. The ability to incorporate a variety of food items is very important since the catfish diet changes dramatically as it ages. Commonly feeding near the bottom of the water body juvenile catfish feed primarily on aquatic insects while adults are more opportunistic going for small crustaceans, aquatic vegetation, and small fish. Fish become a major part of the diet in larger catfish (greater than 18 inches), which can grow to 50 inches (127 cm). Two separate ingestion terms were programmed, one for juveniles and one for adults. Perchlorate levels in food items at Caddo Lake were based on limited data from Smith et al. (2001), however no crustacean data were available. Resulting from a lack of lab and field data for calibration and verification we are unable to test these terms. Appropriate data on ingestion rates, feeding preference, and food item contamination are needed.

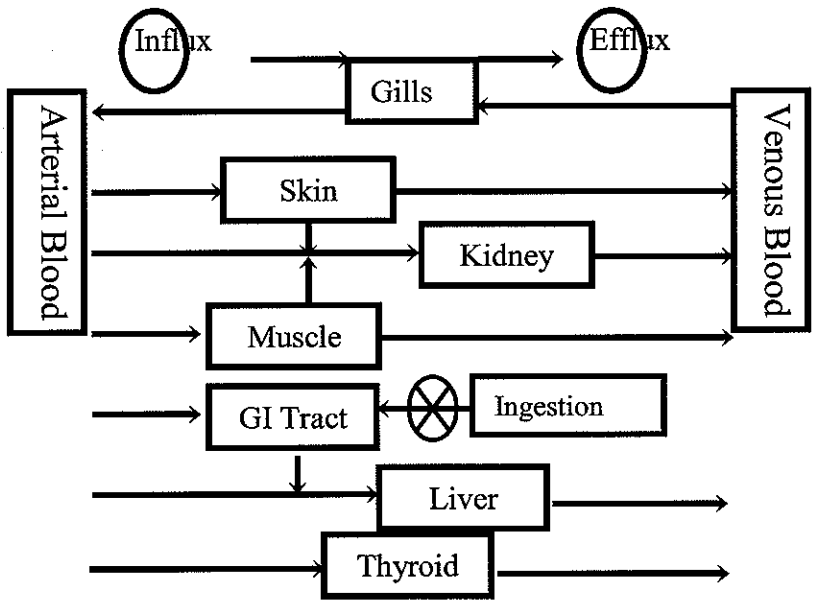

Figure 1. Flow Diagram of the PBTK model for perchlorate uptake in fish.

A six-compartment model of combined T_3 and T_4 kinetics originally developed for mammals (DiStefano 1986; Bianchi et al. 1987; Pilo et al. 1990; Hershman et al. 1986) and later applied to rainbow trout (Sefkow et al. 1996) was used to simulate the secretion of T_3 and T_4 in channel catfish, as well as the impacts of perchlorate on secretion rates. The six compartments represented included: T_4 slow exchange tissue pool, T_4 rapid exchange tissue pool, T_4 plasma pool, T_3 slow exchange tissue pool, T_3 rapid exchange tissue pool, and T_3 plasma pool. The thyroid model was first calibrated by comparing the steady-state mass-balance distribution by percentage to those reported by Sefkow et al.

(1996). Plasma T_3 and T_4 compartments were then adjusted, keeping the same percentage distribution with other compartments, to meet levels reported by Gaylord et al. (2001) in channel catfish.

Hormone inhibition by perchlorate was calculated from data on mosquitofish dosed with sodium perchlorate for 2, 10, or 30 days at doses of 0, 0.1, 1, 10, 100, and 100mg/L (ppm) (Bradford 2002). Whole body T_4 concentrations were determined by radioimmunoassay for pooled groups of fish. A regression curve was fit to the data to derive the inhibition equation based on the concentration of perchlorate in the thyroid tissue. A sensitivity analysis performed on the inhibition term, identified all compartments as insensitive to an increase or decrease of 15%.

Plant Model. The model includes uptake in both terrestrial and aquatic macrophytes. Both models were programmed in Matlab using difference equations. To simulate and predict the uptake and transport of perchlorate in various terrestrial and aquatic plants, we developed new uptake and distribution components that are specific to perchlorate and modified CERES by incorporating these new components (Figure 2). Additionally, an internal hydrological component was added to simulate environmental soil and water conditions.

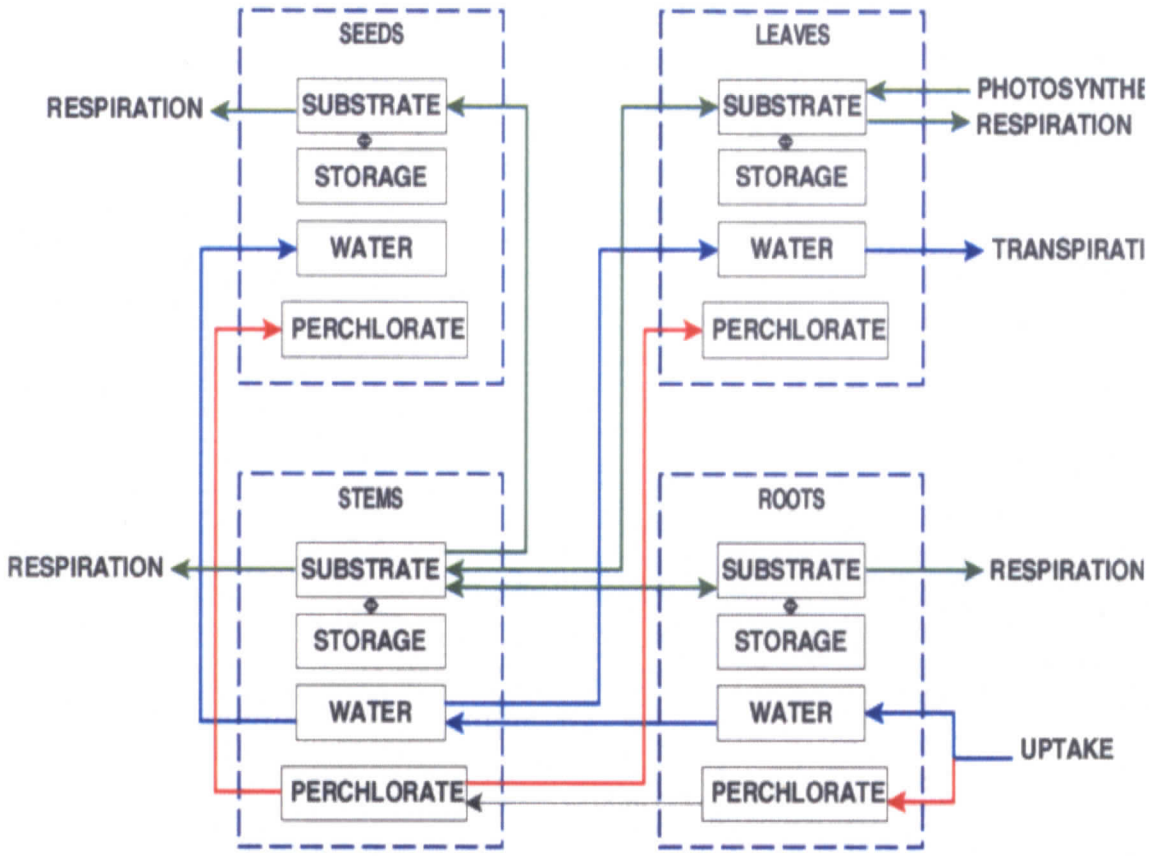

Figure 2. Flow diagram of macrophyte model

For the macrophyte model governing equations, we used those described by Dixon, et al. (1978).

The plant's water uptake is the product of the plant's ability to take up water, its leaf area, and the volume of water available to the plant in its growing soil horizon:

$$U_t^i = f \cdot L'_{(t)} \cdot SM_A$$

where,
U_t^i = incremental water uptake at time (t)
f = water flow constant (h^{-1})
L' = Leaf Area Index
SM_a = Mass of water in Soil Horizon A ($g \cdot m^{-2}$ land area/hour)

Distribution of water and perchlorate between compartments is defined by the difference in water and perchlorate between compartments:

$$F_{ab} = \begin{cases} (W_a - W_b)/r_{ab} & t_1 < t \le t_4 \\ 0 & otherwise \end{cases}$$

where,
F = flux from compartment a to compartment b ($g \cdot m^{-2}$ land area/hour)
W = amount of water in a given compartment ($g \cdot m^{-2}$ land area)
r_{ab} = water flux constant

The amount of perchlorate in individual compartments is defined by:

$$M_{a,t} = W_{a,t} \cdot C_{ClO4^-} \cdot 10^{-3}$$

where
M_a = amount of perchlorate in compartment a at time t ($\mu g \cdot m^{-2}$ land area)
$W_{a,t}$ = mass of water in compartment a at time t ($g \cdot m^{-2}$)
C_{ClO4^-} = ClO_4^- concentration in the incoming water
The ratio of the amount of perchlorate in the compartment to the biomass (wet weight) of the compartment determines the perchlorate concentration:

$$Q_{a,t} = \frac{M_{a,t}}{B_{a,t} + W_{a,t}}$$

where,
$Q_{a,t}$ = concentration of perchlorate in compartment a at time t ($\mu g \cdot g^{-1}$)
$M_{a,t}$ = amount of perchlorate in compartment a at time t ($\mu g \cdot m^{-2}$)
$B_{a,t}$ = biomass of a given compartment ($g \cdot m^2$)
$W_{a,t}$ = mass of water in compartment a at time t ($g \cdot m^2$)

Plant biomass is calculated by summing the soluble and insoluble photosynthate fractions (Dixon, et al. 1978):

$$B_{a,t} = S_{a,t} + ST_{a,t}$$

where,
$B_{a,t}$ = biomass of compartment a at time t ($g \cdot m^2$)
$S_{a,t}$ = sugar substrate in compartment a at time t ($g \cdot m^2$)
$ST_{a,t}$ = plant storage tissue in compartment a at time t ($g \cdot m^2$)

Model Assumptions:

- transport between leaves and stems occurs from the time of bud formation to the time of abscission.
- transport between stems and fruits occurs from the time of net photosynthesis to the time of abscission.
- transport between the stems and roots is assumed to occur throughout the year.

9.0 RESULTS

Small Mammal Model.
We conducted simulations to reflect the lab experiments in MRT-03-01. We assumed that study subjects were exposed to initial ambient temperature for 150 hours, and then were suddenly moved to a cold environment (12°C) and kept there for 50 hours. Air velocity was thought of as zero. A perchlorate correcting coefficient was introduced into the model to estimate the effect of perchlorate on the regulation of metabolic rate and body temperature.

Cold stress:

The predicted results indicate that the individual body regulates its skin temperature to reach equilibrium the fastest (Figure 3). Rectal temperature increased initially when

suddenly moving to cold temperature, and then decreased very slowly to reach equilibrium. At the same time, the sudden cold stress lead to the increase of metabolic rate and the decrease of average body temperature.

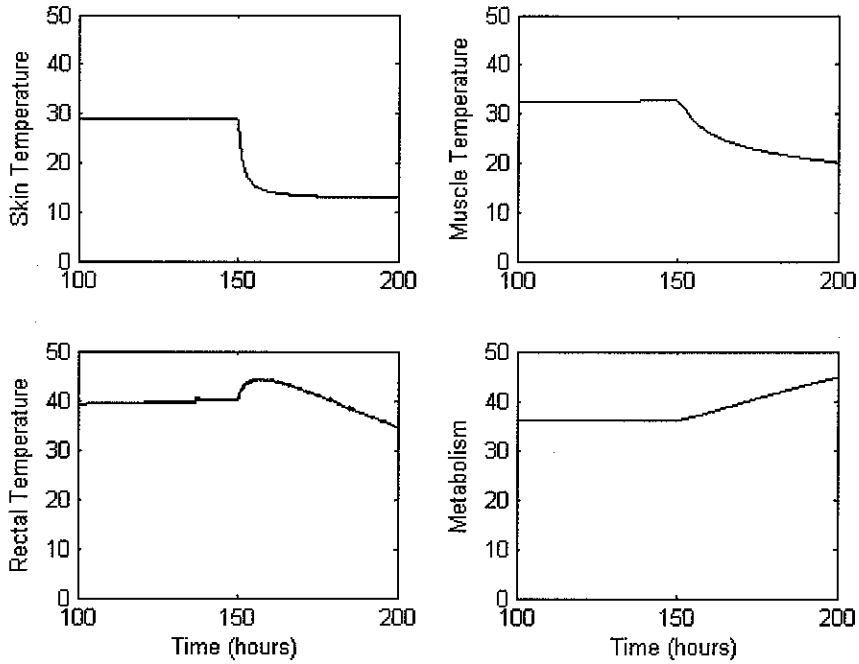

Figure 3. Computer simulation for the transient temperature and vasomotor responses of a subject when exposed to a 16°C decrease in ambient temperature (28°C to 12°C) at 150 hrs (*pf*=1.0).

Perchlorate factor:

Reducing the value of perchlorate factor from 1.0 to 0.6 did not significantly affect the regulation of body temperature, but obviously changed the metabolic rate. The effect of perchlorate exposure (ingestion exposure to perchlorate) decreased the normal metabolic rate, and led to the faster pace of the increase of metabolic rate when suddenly moving to cold stress (Figure 4).

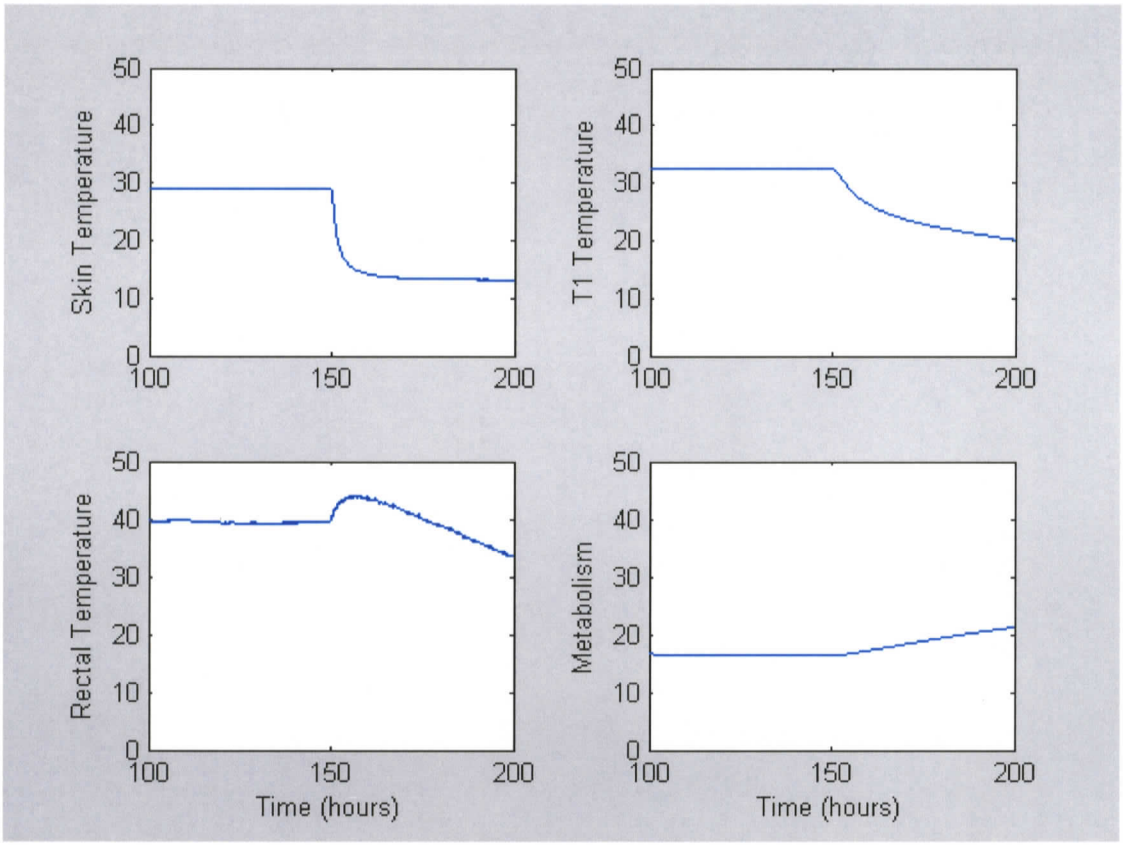

Figure 4. Computer simulation for the transient temperature and vasomotor responses of a subject when exposed to a 16°C decrease in ambient temperature (28°C to 12°C) at 150 hrs (*pf*=0.60).

Fish Model.

Exposure Scenario. A "worst-case" scenario was developed, in which individual catfish were exposed to the maximum effluent perchlorate concentration from the groundwater treatment plant of 975 ppb. Most tissue compartments took approximately 100-200 hours to equilibrate, with the thyroid compartment taking 300 hours (Figures 5, 6). If we assume that 975 ppb is the highest level any fish is exposed to then all measured field tissue concentrations should be less than or equal to those simulated. Currently there are no measured field concentrations in channel catfish to compare these results to for verification. Largemouth Bass collected in Caddo Lake had trace levels or less in muscle and liver tissues, but no data was collected on other tissue compartments (Smith et al. 2001). For representative purposes the model was run a second time using the juvenile ingestion term (Figures 7, 8).

The thyroid concentration data were then used in the hormone secretion model to determine the level of hormone inhibition. Perchlorate inhibition was initiated after 1000 hours to allow the hormone compartments to reach steady-state prior to insult (Figure 9). T_3 hormone levels decreased 17.3-20.8% and T_4 levels decreased 54% (Table 1). No field data have been collected on thyroid hormone levels in any fish species for model verification.

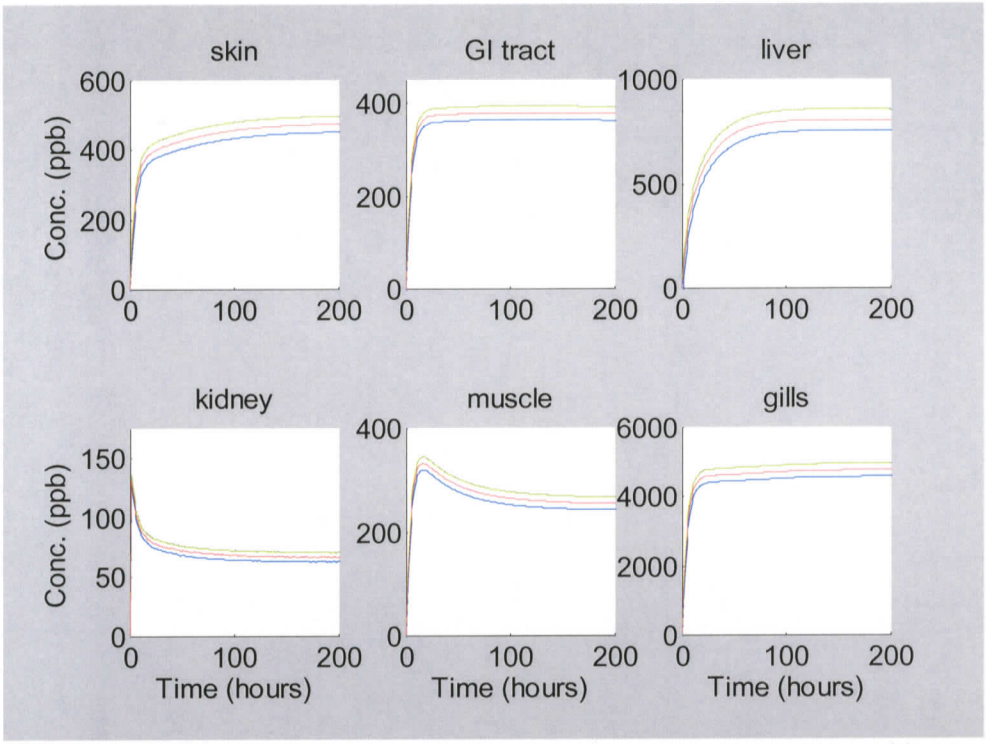

Figure 5. Simulated tissue concentrations based on a 975 ppb dose of perchlorate.

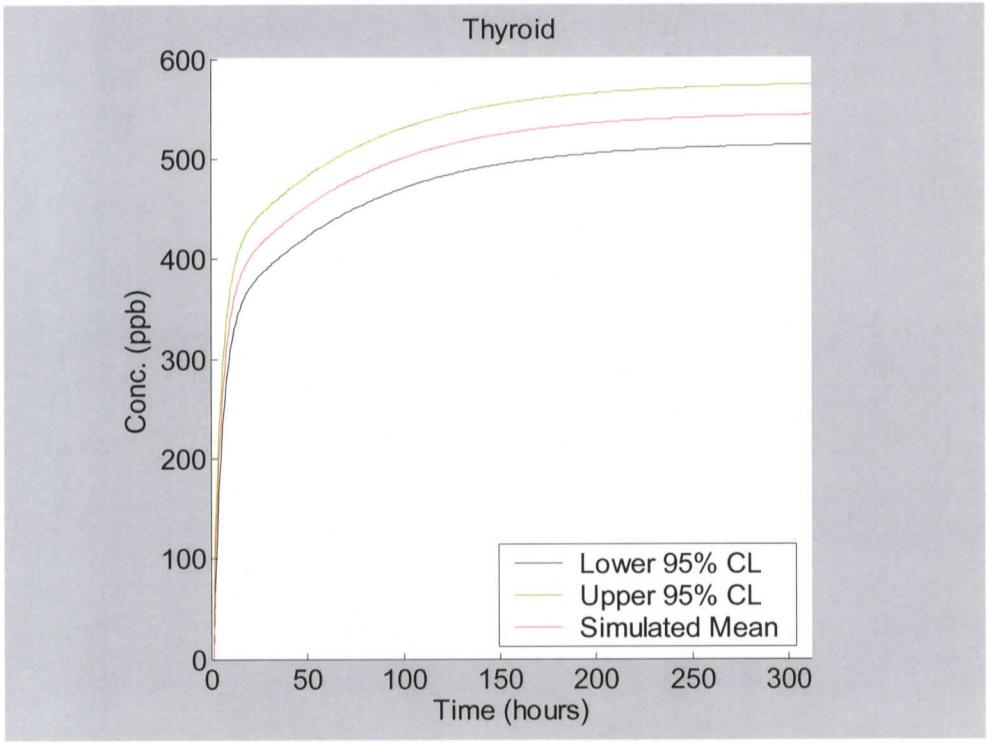

Figure 6. Simulated thyroid tissue concentrations based on a 975 ppb dose of perchlorate.

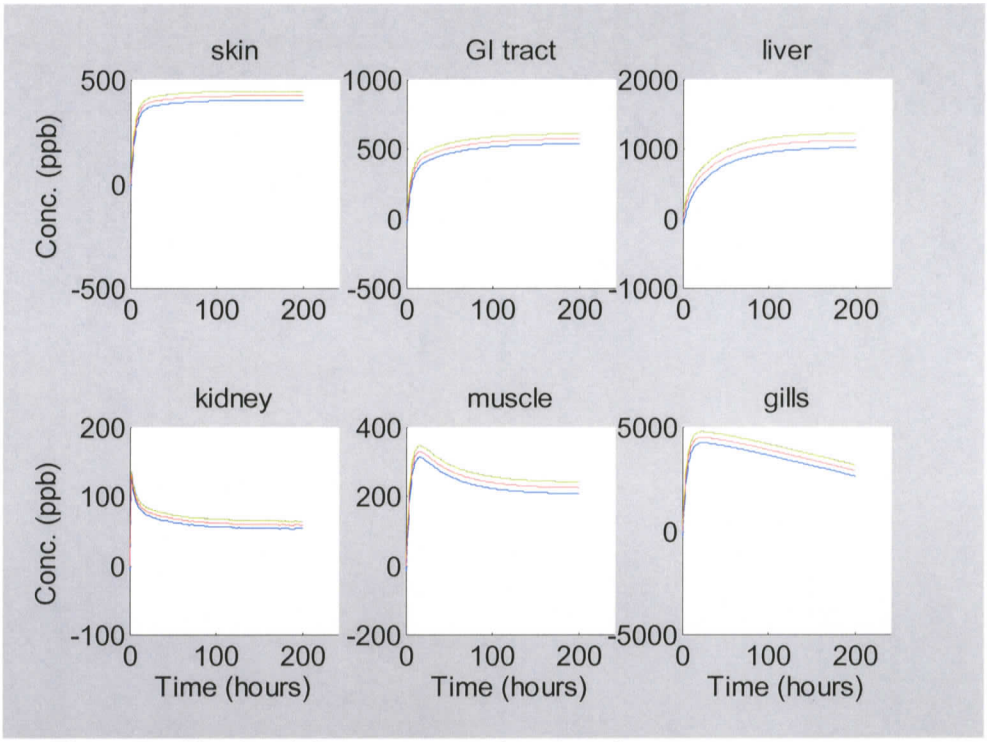

Figure 7. Simulated tissue concentrations based on a 975 ppb dose of perchlorate with juvenile ingestion term.

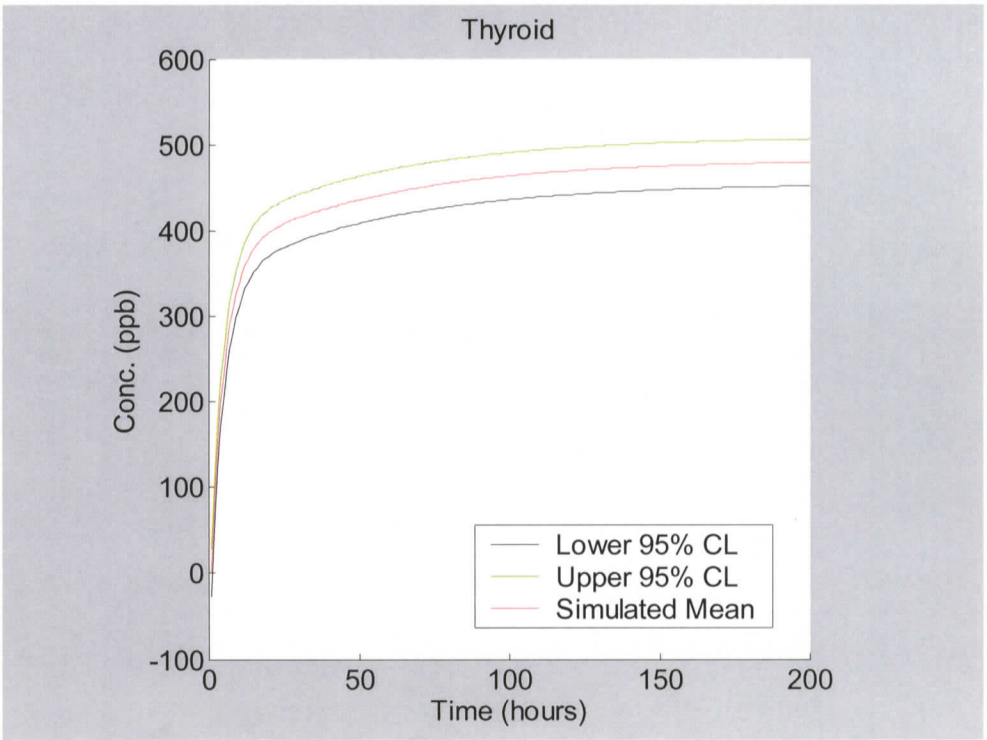

Figure 8. Simulated thyroid tissue concentrations based on a 975 ppb dose of perchlorate with juvenile ingestion term.

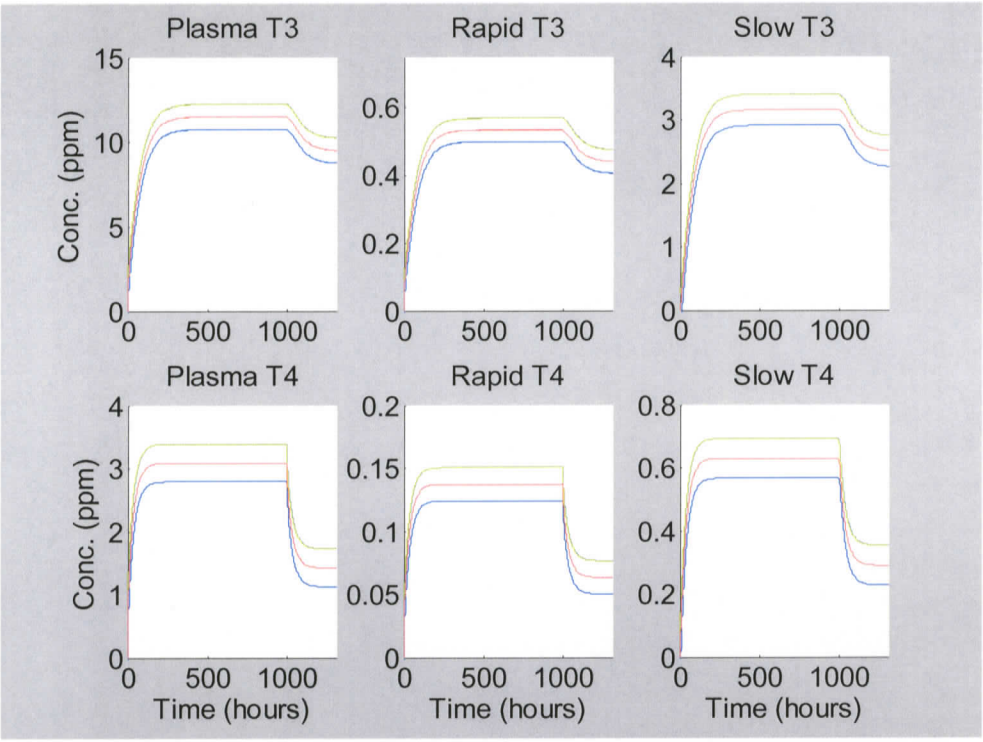

Figure 9. Simulated hormone inhibition based on a 975 ppb dose of perchlorate starting at hour 1000.

Table 1. Thyroid hormone inhibition based on a 975 ppb dose of perchlorate starting at hour 1000.

	Steady State ng/ml	Simulated ng/ml (% decrease)
Plasma T3	11.5	9.4761 (17.6%)
Fast Pool T3	0.5347	0.4401 (17.7%)
Slow Pool T3	3.1605	2.5054 (20.7%)
Plasma T4	3.0908	1.421 (54%)
Fast Pool T4	0.1373	0.0631 (54%)
Slow Pool T4	0.63	0.2897 (54%)

Plant Model.

There are few data available on the partitioning of perchlorate in plant parts. We used data from a study of perchlorate in plants near the Naval Weapons Industrial Reserve Plant near Waco, Texas. Perchlorate concentrations in mulberry leaves and fruits measured during October were 1310±1720 ppb and 467±661ppb respectively (Figure 10). The plant model was calibrated to these data using a water concentration of 47±28 ppb from the same study. The predicted perchlorate concentration was adjusted to fit the

observed data by reducing uptake from the soil water and reducing the flow into leaves and fruits.

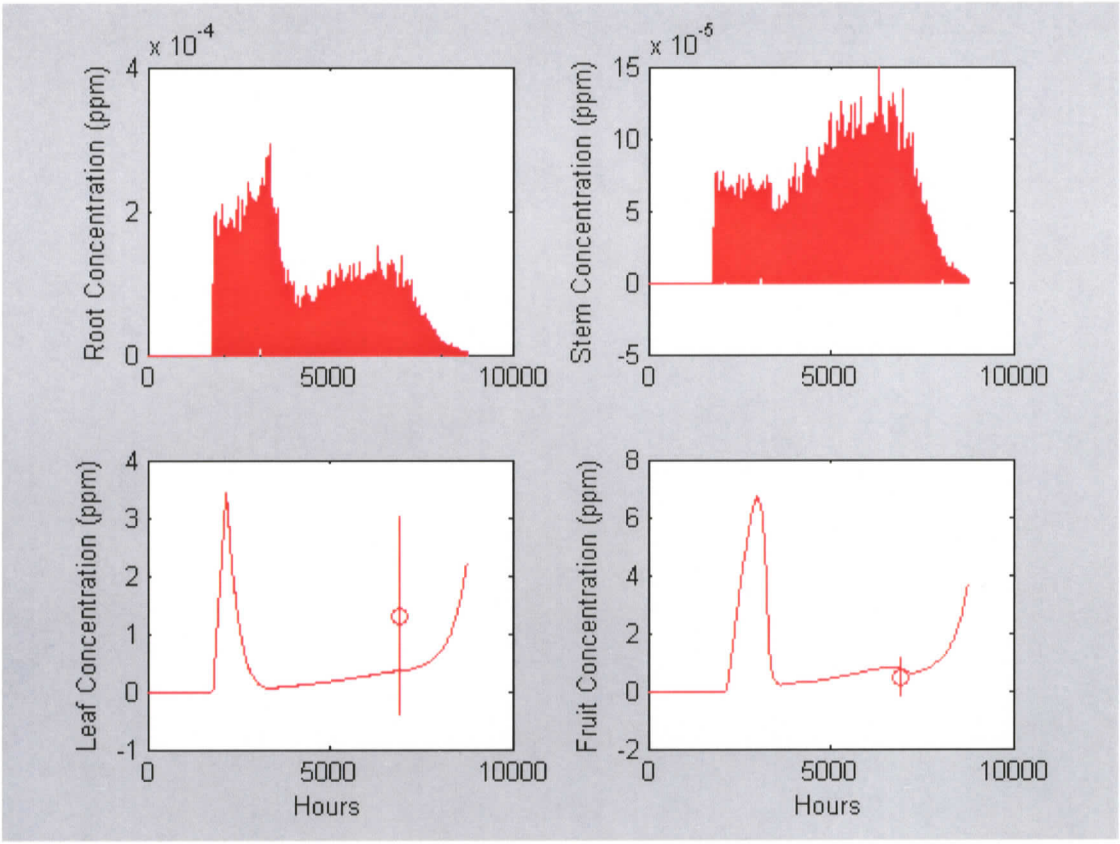

Figure 10. Observed and predicted perchlorate concentrations in mulberry plant parts.

10.0 DISCUSSION

Small Mammal Model.

The modified Crosbie, et al. (1961) model comprehensively simulated the effects of cold stress and perchlorate on the regulation of metabolic rate and body temperature well. Cold stress simulation predicted that ambient temperature is the main factor to affect the regulation of metabolic rate and the body temperature. We can understand that the regulation of body temperature when exposed to cold stress resulted from the change of metabolic rate. In the nude man model (Crosbie, et al. 1961), the vaporization increased as the ambient temperature increased above 28°C, and the metabolic rate increased as the ambient temperature decreased below 28°C. In a cold environment, the increase of metabolic rate is the main physiological mechanism of the regulation of body temperature. Environmental chemicals, such as perchlorate, disturb the function of the thyroid gland that involves body metabolism, and then decreases the body metabolic rate. When suddenly meeting the cold stress, the body has to increase its metabolic burden to keep the normal body temperature.

Although Crosbie, et al.'s model could simulate the regulation of metabolic rate and thermoregulation, the effect of perchlorate should receive further study to identify the mechanisms involved in disturbing thyroid hormone production

Fish Model. The lack of measured field concentrations for catfish tissue concentrations and fish thyroid hormone levels makes it impossible to determine if the current models are accurately simulating perchlorate uptake and effects. It is important to note that the use of the maximum effluent concentration is a true "worst-case" scenario and the actual exposure levels are significantly less. This is evidenced by only trace levels in largemouth bass found in Caddo Lake and a high of 206 ppb in a composite sample of seven mosquitofish taken from the Harrison Bayou Ponded Area, where the GWTP effluent is discharged.

Limited data sets for initial model calibration resulted in a simplification of the thyroid compartment. A singular thyroid compartment may be an oversimplification of the kinetic behavior of perchlorate. Chow and Woodbury (1970) determined that a three-compartment model (stroma, follicle, and lumen) was necessary to properly model the behavior of perchlorate in rat thyroids. It is unknown if fish would follow a similar kinetic behavior since they lack the centralized thyroid gland of mammals. The simplification of the thyroid compartment, in conjunction with the use of 100 ppm dosing data for calibration, instead of a range of concentrations, may have incorrectly estimate uptake at low doses.

The rapid, and substantial, decrease in hormone levels should be viewed cautiously. We do not know if channel catfish respond in the same way to perchlorate exposure as the mosquitofish used for the inhibition term. Additionally we cannot fully characterize the system without the data necessary to determine a T_3 inhibition term, hence the reason T_3 levels decreased substantially less than T_4. At present the simulated results are only a "best guess" and should not be viewed as a definitive answer. It is also important to note that fish are very adept at altering thyroid hormone levels based on various environmental and dietary conditions. As a result of this ability, fish tend to rapidly reestablish normal hormone levels once the inhibitory condition is removed. In addition, various studies have shown that different species rapidly eliminate perchlorate and recover from exposure, once they are removed from a contaminated system.

Plant Model.
The vascular plant model was developed under the assumption that water is the driving force behind the uptake and distribution of perchlorate in plants. Because the model predicts tissue concentrations that are in line with laboratory and field values, it is reasonable to assume that water movement in plants is an important driving force in the uptake and distribution of perchlorate.

The model also indicates that perchlorate is capable of bioaccumulation in the leaves and fruits of exposed plants. If this result is true, there is significant potential for trophic transfer of perchlorate if wildlife and humans consume exposed plants. Although

parameter estimates were based on calibration with lab and field data, direct parameter estimation may improve the accuracy of the model predictions.

11.0 STUDY RECORDS AND ARCHIVE

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one after study completion date.

12.0 REFERENCES

Bianchi, R., G. Iervasi, A. Pilo, F. Vitek, M. Ferdeghini, F. Cazzuola, and G. Giraudi. 1987. Role of serum carrier proteins in the peripheral metabolism and tissue distribution of thyroid hormones in familial dysalbuminemic hyperthyroxinemia and congenital elevation of thyroxine-binding globulin. J. Clin. Invest. 80: 522-534.

Bradford, C. 2002. Perchlorate uptake and effects on thyroid function in fish. Master's Thesis, Texas Tech University, Lubbock, Texas.

Chow S.Y., D.M. Woodbury. 1970. Kinetics of distribution of radioactive perchlorate in rat and guinea-pig thyroid glands. J. Endocrinol., 47:207-218.

Crosbie, R. J., J. D. Hardy, and E. Fessenden. Electrical Analog Simulation of Temperature Regulation in Man. 1961. In "TEMPERATURE: Its Measurement and Control in Science and Industry". Reinhold Publishing Corporation, New York, Vol. 3, p. 627.

DiStefano, J. J., III. 1986. Modeling approaches and models of the distribution and disposal of thyroid hormones. *In* "Thyroid Hormone Metabolism" (Hennemann, G., Ed.). Dekker, New York/Basel.

Dixon, K. R., R. J. Luxmoore, and C. L. Begovich. 1978. CERES - a model of forest stand biomass dynamics for predicting trace contaminant, nutrient, and water effects. I. Model description. Ecol. Modelling 5:17-38.

Erickson, R.J. and J.M. McKim, 1990. A model for exchange of organic chemicals at fish gills: Flow and diffusion limitations. Aquatic. Toxicol. 18: 175-198.

Gaylord, T.G., D.S. MacKenzie and D.M. Gatlin III. 2001. Growth performance, body composition and plasma thyroid hormone status of channel catfish (Ictalurus punctatus) in response to short-term feed deprivation and refeeding. Fish Physiol. Biochem. 24: 73-79.

Hershman, J., K. Nadamanee, M. Sugawara, A.E. Pekary, R. Ross, B. Singh, and J.J. DiStefano, III. 1986. Thyroxine and triiodothyronine kinetics in cardiac patients taking amiodarone. Acta Endocrinol. 111: 193-199.

Machle, W., and T. F. Hatch. 1947. Heat: man's exchange and physiological responses," Physiol. Rev. 27, 200.

Nichols, J.W., J.M., Anderson, M.E., Gargas, M.L., Clewell, H.J., III and R.J. Erickson. 1990. A physiologically based toxicokinetics model for the uptake and disposition of waterborne organic chemicals in fish. Toxicol. Appl. Pharmacol. 106:433-447, 1990.

Nichols, J.W., J.M. McKim, M.E. Anderson, G.J. Lien, A.D. Hoffman and S.L. Bertelson. 1991. Physiologically based toxicokinetics modeling of three waterborne chloroethanes in rainbow trout (*Oncorhyncus mykiss)*. Toxicol. Appl. Pharmacol. 110:374-389.

Nichols, J.W., J.M. McKim, G.J. Lien, A.D. Hoffman, S.L. Bertelsen and C.A. Gallinat. 1993. Physiologically-based toxicokinetic modeling of three waterborne chloroethanes in channel catfish, *Ictalurus punctatus*, Aquatic Toxicol. 27:83-112.

Pilo, A., G. Iervasi, F. Vitek, M. Fereghini, F. Cazzuola and R. Bianchi. 1990. Thyroidal and peripheral production of 3,5,3'-triiodothyronine in humans by multicompartmental analysis. Am. J. Physiol. 258: E715-E726.

Ricklefs, R. E. 1993. The Economy of Nature, third edition. pp.189. W.H. Freeman, New York.

Sefkow, A. J., J. J. DiStefano III, B. A. Himick, S. B. Brown and J. G. Eales. 1996. Kinetic analysis of thyroid hormone secretion and interconversion in the 5-day fasted rainbow trout, *Oncorhyncus mykiss*. Gen. Comp. Endocrinol. 101: 123-138.

Smith P.N., C.W. Theodarakis, T.A. Anderson, and R.J. Kendall. 2001. Preliminary assessment of perchlorate in ecological receptors at Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology 10: 305-313.

Wissler, E. H. 1961. The Mathematical Analysis of Heat Transfer and Temperature Relations in the Human Body," University of Texas Report (March 15).

Wyndham, C. J. and A. R. Atkins, 1960. Approach to the solution of human bio-thermal problem with the aid of an analog computer," Paper No. 27, 3rd Internal Conference on Medical Electronics (July, 1960).

29 MAR 2004

PRELIMINARY ASSESSMENT OF RADIOLABELED IODIDE UPTAKE BY BULLFROG TADPOLE THYROID GLAND: A METHOD DEVELOPMENT AND FEASABILITY STUDY

STUDY NUMBER:	FROG-03-01
SPONSOR:	Strategic Environmental and Research Development Program SERDP Program Office 901 North Stuart Street, Suite 303 Arlington, VA 22203
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, TX 79409-1163
TEST SITE:	Texas Tech University Human Sciences Building Box 42002 Lubbock, TX 79409-2002
RESEARCH INITIATION:	July 1, 2003
RESEARCH COMPLETION:	December 31, 2003

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GOOD LABORATORIES PRACTICES STATEMENT

This study was conducted in accordance with established Quality Assurance Program guidelines and in the spirit of Good Laboratory Practice Standards whenever possible (40 CFR Part 160, August 17, 1989).

Submitted By:

James A. Carr

3/26/04
Date

1.0 DESCRIPTIVE STUDY TITLE: Preliminary Assessment of Radiolabeled Iodide Uptake By Bullfrog Tadpole Thyroid Gland: A Method Development And Feasability Study.

2.0 STUDY NUMBER: FROG-03-01

3.0 SPONSOR:
Strategic Environmental and Research Development Program
SERDP Program Office
901 North Stuart Street, Suite 303
Arlington, VA 22203

4.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:
Start Date: 7/1/03
Termination Date: 12/31/03

6.0 KEY PERSONNEL:
James A. Carr, Co-Principal Investigator/ DBS Testing Facility Management/Study Director
Mike Wages, Research Associate
Mathilde Odeyer, undergraduate research student
James Sullivan, undergraduate research student
Jeff Thatcher, undergraduate research student
Nathan Collie, unpaid consultant
Todd Anderson, Analytical Chemist/ Asst. Director for Science
Ryan Bounds, Quality Assurance Manager
Ronald Kendall, Principal Investigator/ TIEHH Testing Facility Management

7.0 STUDY SUMMARY:

We studied iodide uptake using thyroid cartilage and isolated thyroid glands from bullfrog tadpoles in vitro. In the first set of experiments we attempted to measure rapid uptake of ^{125}I using $^{3}HPEG$ as a tissue extracellular space marker as a means of determining the first order rate kinetics for iodide transport by the sodium-dependent iodide symporter (NIS). There was no apparent time-dependent transport of ^{125}I into the thyroid/cartilage composite, and no affect of perchlorate on calculated ^{125}I transport (100

μM perchlorate). Moreover, cartilage alone exhibited greater apparent iodide transport than thyroid/cartilage complexes together. These results suggested that the thyroid/cartilage composite may be a) non-specifically binding considerable ^{125}I, and b) that $^{3}HPEG$ was not a suitable extracellular space marker.

To avoid the confounding effects of nonspecific binding to cartilage, and the confounding results obtained using $^{3}HPEG$ as an extracellular space marker, we carried out a second set of experiments to investigate the feasibility of using isolated tadpole thyroid glands for determining iodide transport. The results clearly demonstrate that 4 x 15 s washes are sufficient to remove most of the background counts and that ^{125}I uptake by thyroid was much greater than background counts in each experiment. Thus, this protocol appears to be promising for working out the first order rate kinetics of the NIS and the effects of perchlorate on transport rate.

8.0 TEST MATERIALS:

Test Chemical name: sodium perchlorate
CAS number: 7601-89-0
Characterization: X% pure,
Source: Sigma-Aldrich Chemical Company

Test Chemical name: sodium iodide
CAS number: 7681-82-5
Characterization: X% pure,
Source: Sigma-Aldrich Chemical Company

Test Chemical name: $Na^{125}I$
Characterization: 99.5% pure, 106 mCi/mL
Specific activity: 17.4 Ci/mg
Source: Perkin-Elmer Life Sciences Products

Test Chemical name: [1,2-^{3}H]-polyethylene glycol
Characterization: 99.5% pure, 0.5-2 mCi/g
Source: Perkin-Elmer Life Sciences Products

Reference Chemical name: Amphibian Ringer's solution, preincubation solution
CAS number: Not Applicable
Characterization: Deionized water containing the following reagents per 20 L: 101.3 mM NaCl (119.48 g); 3 mM KCl (4.47 g); 3 mM $NaHPO_4$ (8.52 g); 1 mM $MgSO_4$ (2.4 g); 2 mM $NaHCO_3$ (33.6 g); 2 mM $CaCl_2$ (4.4 g), 5 mM glucose (18 g).

Reference Chemical name: Amphibian Ringer's solution, incubation solution
CAS number: Not Applicable
Characterization: Deionized water containing the following reagents per 20 L (except for NaI): 101.3 mM NaCl (119.48 g); 3 mM KCl (4.47 g); 3 mM $NaHPO_4$ (8.52 g); 1 mM $MgSO_4$ (2.4 g); 2 mM $NaHCO_3$ (33.6 g); 2 mM $CaCl_2$ (4.4 g), 5 mM glucose (18 g), 5 μM NaI (0.00015 g/0.2 L). On the day of testing $Na^{125}I$ was added to a final concentration of 0.1-1 μCi/mL (approximately 2E5-2E6 cpm/mL, or 30-300 nM ^{125}I).

9.0 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Perchlorate is known to prevent intake of iodide from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife stability as well as human health. Although the effects of AP on thyroid function of larval frogs has already been examined (Goleman et al., 2002a, b) the direct effects of perchlorate on iodide uptake by tadpole thyroid tissue remains unexplored. Bullfrogs are native to the US and thus represent a useful surrogate for extrapolating to other native ranids. Furthermore they are sufficiently large to permit dissection of the thyroid gland (Figure 1) for analysis of in vivo and in vitro exposures. In this study we investigated the feasibility of using an in vitro thyroid preparation to examine perchlorate-dependent inhibition of thyroidal iodide uptake. This information will be useful in determining the affinity of the anuran sodium/iodide symporter to perchlorate, data which presently do not exist but are critical for appropriate modeling of perchlorate effects on frogs. Virtually nothing is known about the molecular structure or transport kinetics of this transporter in other vertebrates, particularly amphibians. It is important to note that our goal was to accurately characterize first order rate kinetics of the NIS, and not simply examine bulk iodide uptake as has been done in other studies. As such, much of the work completed to date falls under the category of method development, with the long term goal being the collection of reliable data on the transport kinetics that can be used to predict and model perchlorate disruption of thyroid function in these organisms.

10.0 TEST ANIMALS:

Species: *Rana catesbeiana*, American Bullfrog
Strain: wild type
Age: prometamorphic larvae
Number: approximately 100
Source: Charles Sullivan Inc.

11.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

In the first set of experiments we examined the time-dependent transport of ^{125}I using ^{3}H-PEG as an extracellular space marker. The goal of these preliminary experiments was to identify an incubation time at which the extracellular space is fully occupied but transport of ^{125}I is till linear and is not yet showing signs of saturation. A total of six experiments were carried in the summer and early fall of 2003. Thyroid glands and associated cartilage from three tadpoles per experiment (Fig. 1) were incubated separately in the presence of $Na^{125}I$ for varying times to determine time-dependent iodide transport. At the incubation time providing maximal transport, thyroid/cartilage complexes were incubated with $Na^{125}I$ in the presence or absence of sodium perchlorate (100 μM).

The results from the first studies suggested that co-incubation of thyroid glands and cartilage together would not be feasible. Therefore, we examined the feasibility of examining ^{125}I uptake in isolated tadpole thyroid glands. Rather than use ^{3}H-PEG as an extracellular space marker for determining nonspecific binding, we designed a set of experiments to examine timed rinses of the tissue with ice-cold preincubation buffer. Thyroid gland pairs (dissected free from associated cartilage) from three tadpoles per experiment (Fig. 1) were incubated separately in the presence of $Na^{125}I$ for 30 min and washed for varying times with ice-cold preincubation buffer.

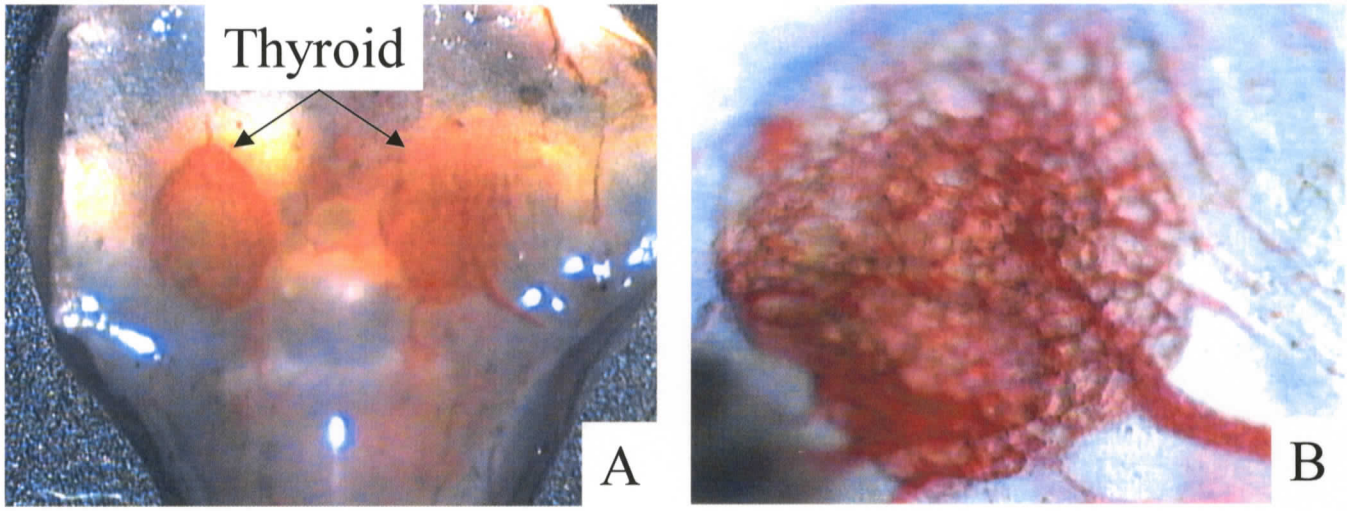

Figure 1. Microdissected thyroid glands from prometamorphic bullfrog tadpoles. Fig. 1A. Thyroid glands attached to branchial cartilage. The thyroid glands exist as distinct bilaterally symmetric pair of glands attached to the cartilage by connective tissue. Fig. 1B. Enlargement of a single thyroid gland showing the follicular structure and highly vascularized nature of the gland. Photo taken by Dr. Carr at Elms College, Chickopee MA, February 2003.

12.0 METHODS:

12.1 Test System acquisition, quarantine, acclimation. Prometamorphic tadpoles (Taylor-Kollros stage XV-XIX) were obtained from Carolina Biological Supplies. Refer to TIEHH AQ-1-14 for details on routine *R. catesbeiana* tadpole husbandry. They were maintained in a large fiberglass enclosure (760 L, 108"L x 24"W x 22"D, stocking density = 50) in filtered and dechlorinated tap water at 22 $\pm$ 2 °C on a 12L: 12D light regimen for at least 7 d prior to initiation of exposure. The tank was labeled as indicated in SOPs TIEHH AQ-1-24/ DBS ET-1-03, which includes genus and species name, common name, project name, number, and start date, and the name of the person primarily responsible for animal care

12.2 Test Material Application
Rates/concentrations: 100 ppb
Frequency: once for 2 min

Route/Method of Application: Perchlorate was added to the incubation medium bathing the thyroid tissue.

12.3 Animal Sacrifice and Sample Collections. Animals were quickly euthanized in MS-222 (1g/L, TIEHH AQ-1-03/ DBS AF-3-03). In the first set of experiments using the radiolabeled extracellular space marker ^{3}HPEG, thyroid glands attached to the branchial cartilage were removed. In a second set of experiments, isolated thyroid gland pairs were removed. Tissues were placed in 1 mL of ice-cold oxygenated preincubation buffer. After all tissues were collected, they were transferred to individual wells of a sterile 24-well culture plate containing 1 mL of prewarmed, oxygenated preincubation buffer at 21° C. Tissues were pre-incubated for 5 min before transfer to individual wells containing 1 mL of incubation buffer and $Na^{125}I$ (and ^{3}HPEG in some experiments). Tissues were then incubated for varying periods of time at 21° C and constant gassing with 95% O_2/ 5% CO_2 in a Dubnoff shaker. At the end of the incubation, tissues were blotted. In experiments in which ^{3}HPEG was used, tissues was transferred to scintillation vials containing Soluene. Tissues were solubilized at 60° C for 16 h followed by liquid scintillation spectroscopy. In cases where ^{3}HPEG was not used, tissues were transferred directly to 12 x 75 mm polypropylene culture tubes and counts determined on a gamma counter.

12.4 Endpoint Analysis
Counts per minute was the measured endpoint.

13.0 RESULTS:

In February 2003, Dr. Carr visited the laboratory of Sr. Dr. Mary Wright at Elm's College in Chickopee MA to learn the method for dissecting thyroid glands from bullfrog tadpoles. The methodology was modified during this visit and it was clear after examining several developmental stages that the thyroid glands were most easy to remove and were largest in prometmamorphic tadpoles between the stages of XV and XXII. After stage XXII the thyroid glands move laterally and lose quite a lot of their

vascularization, making them very difficult to visualize in fresh preparations. The large size of thyroid glands between stages XV and XXII corresponds to the intense degree of thyroid hormone secretion that is taking place during this period as tadpoles lose their tails and the forelimbs emerge.

Results of the initial transport studies are reported in Table 1. In these experiments, ^{3}HPEG was used as a tissue extracellular space marker, and counts present in the extracellular apace were subtracted from total counts to obtain specific ^{125}I transport in fmol/min. There was no apparent time-dependent transport of ^{125}I into the thyroid/cartilage composite, and no affect of perchlorate (100 μM perchlorate) on calculated ^{125}I transport. Moreover, cartilage alone exhibited greater apparent iodide transport than thyroid/cartilage complexes together. These results suggested that the thyroid/cartilage composite may be a) non-specifically binding considerable ^{125}I, and b) that ^{3}HPEG was not a suitable extracellular space marker.

In the first set of experiments, the size of the branchial cartilage relative to the size of the thyroids was considerable (Fig. 1). To avoid the confounding effects of nonspecific binding to cartilage, and the confounding results obtained using ^{3}HPEG as an extracellular space marker, we carried out a second set of experiments to investigate the feasibility of using isolated tadpole thyroid glands for determining iodide transport. To our knowledge this has never been done before, and we wanted to confirm that we could detect measurable transport. In order to confirm that we were maximally reducing background counts and could still detect measurable ^{125}I uptake by thyroids, we incubated isolated thyroid gland pairs with ^{125}I for 30 min and then examined the washout kinetics using cold preincubation buffer. The results of two washout experiments are shown in Figures 2 and three. In these experiments each wash lasted 60 s (Experiment 1) or 15 s (experiment 2). The results clearly demonstrate that 4 x 15 s washes are sufficient to remove most of the background counts and that ^{125}I uptake by thyroid was much greater than background counts in each experiment. Thus, this protocol appears to be promising for working out the first order rate kinetics of the NIS and the effects of perchlorate on transport rate. We had planned to conduct experiments along these lines in late November but were unable to get prometamorphic tadpoles at the right developmental stage. We are waiting for these animals to develop to later prometamorphic stages to continue testing this protocol.

Table 1. Mean (± SEM) specific iodide transport in thyroid/branchial cartilage composites from bullfrog tadpoles.

Treatment		Specific iodide transport (fmol/min)*
5 μM NaI		
	2 min	7.59 ± 2.55
	8 min	2.16 ± 0.61
	16 min	1.50 ± 0.20
	30 min	1.24 ± 0.11
5 μM NaI + 100 μM perchlorate		
	2 min	2.55 ± 1.02
5 μM NaI + 100 μM perchlorate (Cartilage alone)		
	2 min	12.2 ± 2.85

* Determined by subtracting ^{3}HPEG counts from total counts.

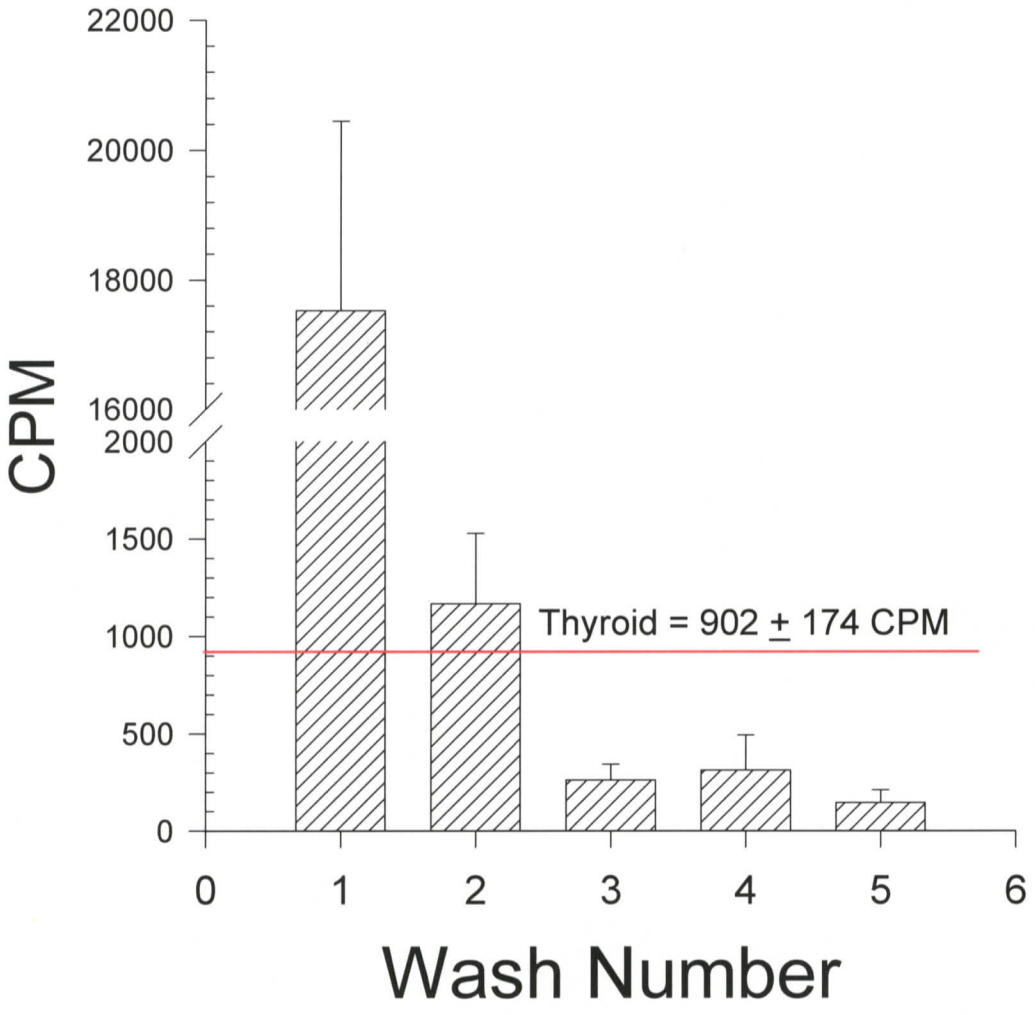

Figure 2. Results of the first washout experiment on isolated thyroid pairs from prometamorphic bullfrog tadpoles. Total counts per minute (CPM) in the incubation medium are shown on the y-axis. Wash times were 60 s each. Paired thyroid glands from three tadpoles were incubated individually with ^{125}I for 30 min at 21° c with constant 95%O_2/5%CO_2 gassing. The ^{125}I (mean CPM per thyroid pair) taken up by the paired thyroids is shown by the red line.

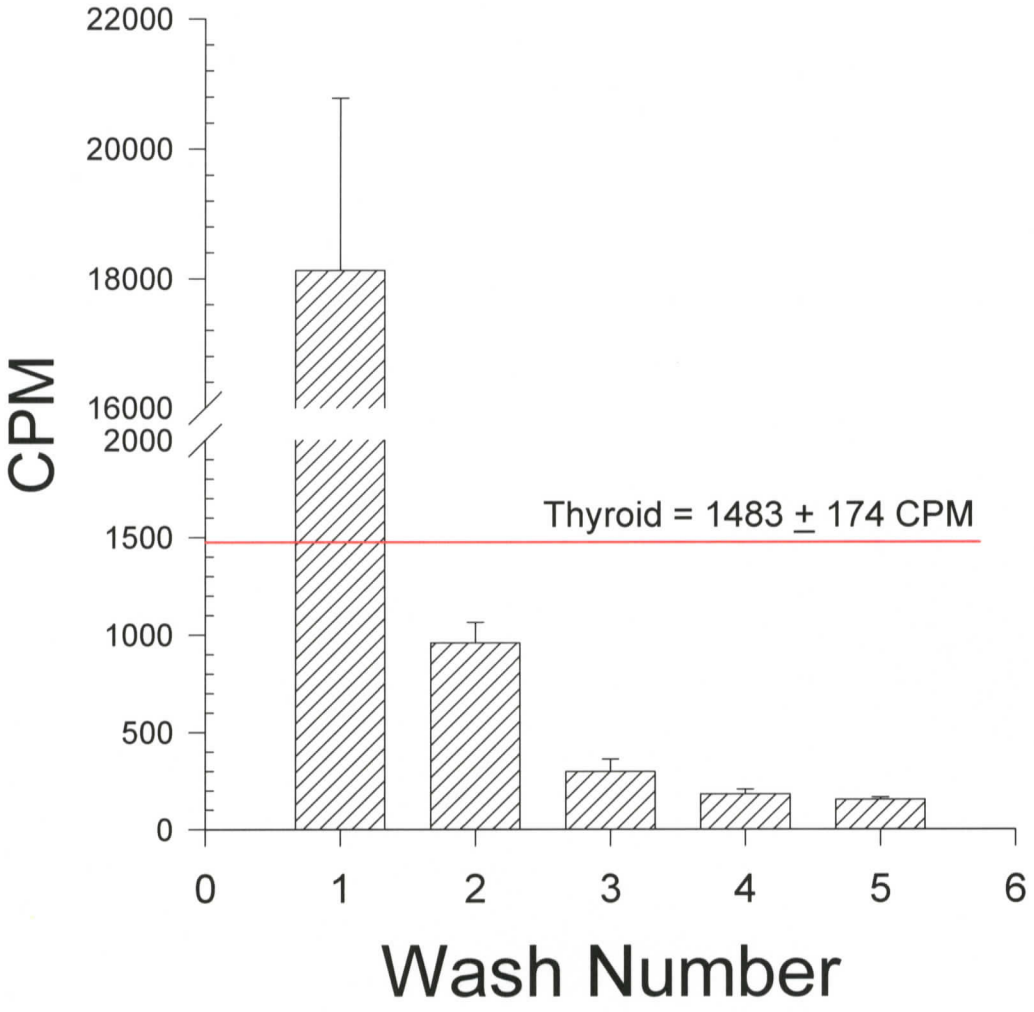

Figure 3. Results of the second washout experiment on isolated thyroid pairs from prometamorphic bullfrog tadpoles. Total counts per minute (CPM) in the incubation medium are shown on the y-axis. Wash times were 15 s each. Paired thyroid glands from three tadpoles were incubated individually with ^{125}I for 30 min at 21° c with constant 95%O_2/5%CO_2 gassing. The ^{125}I (mean CPM per thyroid pair) taken up by the paired thyroids is shown by the red line.

10. DISCUSSION:

Our data indicate that thyroid glands must be isolated prior to incubation in order to minimize the confounding effects of nonspecific background binding of ^{125}I to cartilage. The data on iodide uptake in isolated, paired thyroids is highly reproducible. The confounding effect of nonspecific ^{125}I uptake was so great in the first series of experiments that we observed no effect of perchlorate on ^{125}I uptake. Perchlorate is well known to have great affinity for the NIS.

Our data also indicate that not all developmental stages are suitable for thyroid studies in vitro. The thyroid glands are extremely difficult to locate in adult frogs and in early stage tadpoles because the degree of vascularization is quite a bit less than during late prometamorphosis, when the thyroid is at its greatest level of activity. This creates somewhat of a problem in obtaining suitable numbers of tadpoles at the right developmental stages, because two commercial suppliers do not adequately stage their material that they provide and another commercial source (Charles Sullivan Inc.) collects specimens directly from local ponds and generally cannot supply animals between the months of October and April. For example, of 50 animals ordered in late 2003, only 2-3 were of the correct developmental stage. Although these animals continue to grow, it may be several weeks before they reach prometamorphosis. Bullfrog tadpoles may take 2-3 years total to complete metamorphosis.

11. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

12. REFERENCES:

Goleman,W.L., Urquidi, L.J., Anderson, T.A., Kendall, R.J., Smith E.E., and Carr, J.A. (2002a). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Goleman, W.L., Carr, J.A., and Anderson, T.A. (2002b). Environmentally relevant concentrations of ammonium perchlorate inhibit thyroid function and alter sex ratios in developing *Xenopus laevis*. Environ. Toxicol. Chem. 21:590-597.

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43:247-249.

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499:787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo renarum* larvae kept in potassium perchlorate solution. *Biocell* 20: 147-153.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63:162-168.